University of Alberta

Prediction of Deoxynivalenol Content in Wheat and Barley Using Near Infrared Reflectance Spectroscopy

by

Lori Anne Oatway ©

A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Master of Science

in

Animal Science

Department of Agricultural, Food and Nutritional Sciences

Edmonton, Alberta
Fall 2008

Library and Archives Canada

Published Heritage Branch

395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque et Archives Canada

Direction du Patrimoine de l'édition

395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence
ISBN: 978-0-494-47379-5
Our file Notre référence
ISBN: 978-0-494-47379-5

NOTICE:
The author has granted a non-exclusive license allowing Library and Archives Canada to reproduce, publish, archive, preserve, conserve, communicate to the public by telecommunication or on the Internet, loan, distribute and sell theses worldwide, for commercial or non-commercial purposes, in microform, paper, electronic and/or any other formats.

The author retains copyright ownership and moral rights in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

AVIS:
L'auteur a accordé une licence non exclusive permettant à la Bibliothèque et Archives Canada de reproduire, publier, archiver, sauvegarder, conserver, transmettre au public par télécommunication ou par l'Internet, prêter, distribuer et vendre des thèses partout dans le monde, à des fins commerciales ou autres, sur support microforme, papier, électronique et/ou autres formats.

L'auteur conserve la propriété du droit d'auteur et des droits moraux qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

In compliance with the Canadian Privacy Act some supporting forms may have been removed from this thesis.

While these forms may be included in the document page count, their removal does not represent any loss of content from the thesis.

Conformément à la loi canadienne sur la protection de la vie privée, quelques formulaires secondaires ont été enlevés de cette thèse.

Bien que ces formulaires aient inclus dans la pagination, il n'y aura aucun contenu manquant.

Canada

Dedication

I am dedicating this thesis to my family who was so supportive and patient during this process.

To Ward, Ezri and Brie.

Abstract

As long as current agricultural practices cannot prevent *Fusarium* infection in small grains, deoxynivalenol (DON) will continue to be a critical issue in animal and human health. The purpose of this project was to determine if near infrared reflectance spectroscopy (NIRS) can predict DON concentrations in cereal grain. First, the NIRS absorption spectrum of DON was isolated and used to develop a baseline calibration using specific wavelength ranges and standard samples developed from commercial flour and pure DON. The second part of this project transferred the baseline calibration to naturally-infected samples of ground wheat and barley. The best equation developed had a coefficient of determination (R^2) of 0.90, standard error of calibration (SEC) of 0.64 ppm, and standard error of cross validation (SECV) of 0.72 ppm. The equations developed in this study demonstrate that NIRS can effectively predict DON content in wheat and barley.

Acknowledgements

I would like to thank:

Dr. Jim Helm for all his guidance, support and mentoring throughout my career and this project, without him I would never have started this adventure;

Dr. Ruurd Zijlstra for all his guidance and helpful suggestions and for being co-supervisor of my program;

Dr. Feral Temelli and Dr. Jim Unterschultz for reviewing this thesis;

Carla Weidner for keeping me organized and helping with this project, Dr. Kequan Xi and Zhanna Hartman for all their help and expertise;

Alberta Agriculture and Rural Development, Alberta Crop Industry Development Fund for providing financial support; and Rahr Malting for their technical support of this project.

My family and the staff at the Field Crop Development Centre in Lacombe for their support and encouragement.

Table of Contents

Dedication

Abstract

Acknowledgements

List of Figures

List of Tables

List of Abbreviations

Chapter 1

Background Information

1.0 Introduction ---------- 1

1.1 Implications of Deoxynivalenol ---------- 3

1.1.1 Fusarium and Deoxynivalenol ---------- 3

1.1.2 Deoxynivalenol - Animal Health and Production ---------- 5

1.1.3 Deoxynivalenol - Food Processing and Human Health ---------- 8

1.2 Economic Impact of DON ---------- 10

1.3 Limits for DON Content ---------- 11

1.4 Detection of Deoxynivalenol ---------- 14

1.5 Near Infrared Reflectance Spectroscopy (NIRS) ---- 18

1.5.1 NIRS and DON ---- 20

1.6 Current State of Knowledge ---- 23

1.6.1 Gaps in Knowledge ---- 24

1.7 Scope of Thesis ---- 24

1.8 Summary ---- 25

References ---- 27

Chapter 2

Isolation of Near Infrared Absorption Peaks for Deoxynivalenol ---- 44

2.1 Introduction ---- 44

2.2 Materials and Methods ---- 46

2.2.1 NIRS Scanning ---- 46

2.2.2 Scanning of Pure DON ---- 47

2.2.3 Calibration Set Development ---- 48

2.2.4 Development of the Calibration Equations ---- 49

2.3 Results and Discussion ---- 50

2.3.1 Pure DON Calibration Analysis ---- 50

2.3.2 Calibration Development on the Dilution Set ---- 54

2.4 Conclusions ---- 65

2.5 References ---- 66

Chapter 3

Using Near Infrared Spectroscopy and Modified Wavelength Ranges to Predict Deoxynivalenol Levels in Naturally Infected Wheat and Barley ---- 69

3.1 Introduction ---- 69

3.2 Materials and Methods ---- 70

3.2.1 Samples ---- 70

3.2.2 NIRS Reflectance and Model Development ---- 72

3.2.3 Development of Calibration Equations ---- 72

3.2.4 Gas Chromatography with Electron Capture Detection ---- 73

3.2.4.1 Sample Preparation ---- 73

3.2.4.2 GC Apparatus ---- 74

3.3 Results and Discussion ---- 74

3.3.1 Validation ---- 81

3.4 Conclusions ---- 85

3.5 References ---- 86

Chapter 4

Project Summary ---- 88

References ---- 93

List of Figures

Figure 1.1. The chemical structure of deoxynivalenol, a type B trichothecene. ...4

Figure 1.2. The chemical structure of 3-acetyl Deoxynivalenol (A) and 15-acetyl Deoxynivalenol (B). 7

Figure 2.1. Absorption spectra from 400 to 2498 nm of an empty sample cup with a black rubber back. 51

Figure 2.2. Absorption spectra from 400 to 2498 nm of the same sample cup containing 5 mg of pure DON. 51

Figure 2.3. Absorption spectra of DON from 400 to 2498 nm. 52

Figure 2.4. PCA classification of 24 ground wheat samples with 0% (A), 6.937% (B), 13.875% (C) and 27.75% (D) DON projected onto PC1 and PC2. 55

Figure 2.5. The absorption spectra (400 to 2498 nm) for the dilution samples using log (1/R) (A), and a first derivative math treatment (B). 57

Figure 2.6. Wavelength ranges (A, 1100 to 1250 nm; B, 1670 to 1720 nm; C, 2100 to 2200; D 2250 to 2350 nm) magnified to show the variation between the standards with different DON concentrations. 58

Figure 2.7. Optimal NIRS calibration equation for DON concentration using, 150 wavelengths from 1100 to 1250, 1670 to 1720 and 2100 to 2200 nm. 62

Figure 3.1. Distribution of calibration samples based on DON content determined by GC analysis. 75

Figure 3.2. Absorption scans of the 141 samples used for DON calibration.....76

Figure 3.3. Calibrations developed for DON content for equation 1 (A), equation 2 (B), equation 3 (C) and equation 4 (D).....78

Figure 3.4. Validation of the developed equations using equation 1 (A), equation 2 (B), equation 3 (C) and equation 4 (D).....82

Figure 3.5. Prediction of the validation group using equation 4; before (A) and after (B) removal of the one sample containing 7.10ppm DON concentration.....83

List of Tables

Table 1.1. Limits for deoxynivalenol content proposed by the EU Commission for various cereals and cereal products. ---------- 12

Table 1.2. Maximum tolerable levels for DON in animal feeds in different nations.---------- 13

Table 2.1. Statistical data for DON calibration models using different wavelength ranges. ---------- 60

Table 3.1. Summary of calibration statistics for modified PLS models using full and reduced wavelengths. ---------- 77

Table 3.2. Summary of validation statistics for modified PLS models using full and reduced wavelengths for calibration---------- 81

List of Abbreviations

DON	Deoxynivalenol
ELISA	Enzyme-linked immunosorbent assay
g	Grams
GC	Gas chromatography
HPLC	High performance liquid chromatography
mg	Milligrams
ppb	Parts per billion
ppm	Parts per million
min	Minutes
MPLS	Modified partial least squares
NIRS	Near infrared reflectance spectroscopy
NIT	Near infrared transmission
PCA	Principle component analysis
R^2	Coefficient of determination
SEC	Standard error of calibration
SECV	Standard error of cross validation
SEP	Standard error of prediction
SEPCV	Standard error of prediction cross validation
TLC	Thin layer chromatography

Chapter 1

Background Information

1.0 Introduction

With the escalating mobilization of feed and food throughout the world, increasing emphasis is placed on food safety and testing of crops. Up to 25% of the crops produced in the world are contaminated with mycotoxins (Rotter et al., 1996). Mycotoxin contamination is cause of great concern because of the variety of toxic effects on humans and animals. Mycotoxins are secondary metabolites produced by fungi and molds that are common contaminants of agricultural crops (Bennett and Klick, 2003). Mycotoxins can be acutely or chronically toxic to humans and animals depending on the kind of toxin and dose (Charmley et al., 1995; D'Mello and Macdonald, 1997). More than 31,000 different fungal metabolites were identified by 2005 and more than 300 different mycotoxins have been differentiated (Biner et al., 2007).

Deoxynivalenol is one of the toxins detected most frequently worldwide and at the highest concentrations (Clear and Patrick, 2000). The production of deoxynivalenol (DON) is primarily associated with *Fusarium graminarum* and *F. culmorum*; both are plant pathogens affecting cereal grains (Desjardins et al., 2001). Corn, barley, wheat, oat, and rye are the grains predominately affected, although rice, sorghum, and triticale can also be infected. Fungal infection, and therefore toxin production, is dependent on a wide variety of conditions. High moisture content, warm temperatures and high humidity all promote fungi production (Manitoba Agriculture, 2007). The *Fusarium* pathogen infects

cereal grains during anthesis and the weather conditions during this period can alter the severity and occurrence of infection (Larsen et al., 2004). The degree of fungal growth can also be affected by soil treatment, crop rotation, and other agronomic treatments (Oldenburg et al., 2000). *Fusarium* species differ in their optimum conditions for growth and infection, causing changes in the type of infections between seasons and across regions (Larsen et al., 2004).

Mycotoxin formation can occur in the field or during storage, so grain that was harvested toxin free, can develop a problem during storage. *Fusarium* infection can reduce kernel set and result in reduction of yield, weight, and quality (Larsen et al., 2004). Fungi can also produce more than one type of toxin. A mycotoxin will not likely occur in isolation and usually a combination of toxins will be present making detection and isolation of specific toxins more difficult (Charmley and Trenholm, 2000).

In western Canada, the first report of widespread contamination of wheat by DON was in 1985 in the wheat crop in Manitoba and eastern Saskatchewan, and it has appeared sporadically during the last 20 years, slowly creeping further west. In 2001, 84% of Manitoba fields and 43 to 50% of Saskatchewan fields were infected with some level of *Fusarium* head blight (Fernandez et al., 2002; Gilbert et al., 2002). *Fusarium* infection is endemic in Manitoba and the disease will reveal itself under the right conditions. The amount of infection is dependent on weather and moisture levels during the growing season; thus, the amount of infection will vary from year to year. In 2006, the levels of *Fusarium* infection were the lowest since 1993, when crop surveys started monitoring infection

levels (Cochrane, 2007). Endemic occurrence of *Fusarium* infection and yearly variation necessitates accurate methods of detecting mycotoxin presence.

While the presence of toxigenic fungus can indicate a potential hazard, only the detection of toxin is useful. Even if the fungus is present, toxin production is not required for growth or reproduction and may therefore not occur (Fink-Gremmels, 1999; Desjardins et al., 1993). Among studies, the relationship between disease incidence or severity and mycotoxin concentrations has not been consistent. Most studies reported a positive correlation (Bai et al., 2001; Cromey et al., 2002; Miedaner et al., 2003) but the amount of toxins per unit disease index differed considerably. Some researchers found that infection and toxin production were not significantly related (Zhou et al., 2002). To further confound the relationship, the toxin may exist in the substrate while the fungus may have disappeared, making the detection of the fungus unreliable (Fink-Gremmels, 1999). The most limiting factor in adequately managing DON infected grain, is the means to detect DON quickly and economically and therefore limit the amount of DON entering into the food chain.

1.1 Implications of Deoxynivalenol

1.1.1 Fusarium and Deoxynivalenol

Fusarium is a large genus of filamentous fungi that contains several strains of economically important pathogenic species. The most important species of *Fusarium* in northern temperate regions are *Fusarium*

graminarum and *Fusarium culmorum* and these species are widely distributed on plants and in the soil (Placinta et al. 1999). These strains cause *Fusarium* head blight in wheat and barley and ear rot in maize (Placintă et al., 1999). *Fusarium* is capable of producing fumonisims, A and B trichothecenes (including DON, 3-acetyldeoxynivalenol and 15-acetyldeoxynivalenol) and zearalenone (Placinta et al., 1999). The pattern and amount of toxin produced can vary within strains of distinct fungal species and from year to year (Fink-Gremmels, 1999).

Premature and uneven ripening in addition to abnormalities in the kernels characterizes a *Fusarium* infection in wheat and barley. Infected seed has signs of shriveling and discoloration and can be lighter in color (tombstone kernels) or show signs of pink discoloration (Richard, 2007). The pink staining is also referred to as 'pink scab'.

One of the most frequently detected *Fusarium* toxins in cereal crops is DON, which is a type B trichothecene (Rotter et al., 1996). The structure of DON is shown in Figure 1.1.

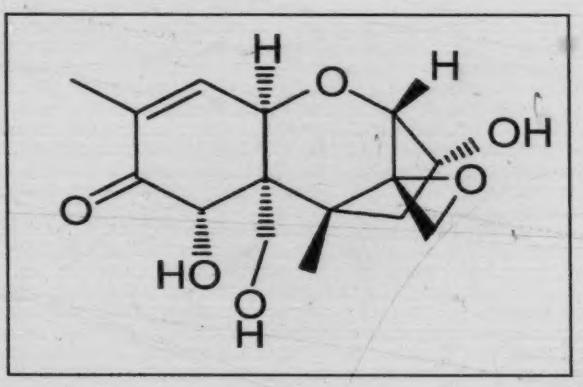

Figure 1.1. The chemical structure of deoxynivalenol, a type B trichothecene.

Deoxynivalenol has a molecular weight of 296.32 and is one of the more polar trichothecenes. Trichothecenes usually contain an epoxide ring at C-12 and C-13 and a double bond at position C-9 and C-10 (Desjardins et al., 1993). The structures of trichothecenes are chemically stable to heat and at neutral to acidic pH values, which allows the toxin to withstand degradation during processing and after ingestion (Krska et al., 2007; Lauren and Smith, 2001).

Japanese scientists first isolated DON in 1972 (Morrooka et al., 1972). The chemical structure was described as 12, 13 – epoxy-3α, 7α, 15-trihydroxy-trichothec-9-ene-8-one and it was named 4-deoxy-nivalenol.

1.1.2 Deoxynivalenol - Animal Health and Production

Mycotoxin contamination of feed ingredients affects the agriculture economy by lowering animal performance and causes food safety concerns with contaminated animal products (Abramson et al, 1997). All animal species evaluated in literature have shown adverse effects from DON ingestion or inhalation, with swine showing the most sensitivity to the toxin and ruminant species being the most resistant (Prelusky et al., 1994).

The initial response to DON exposure in animals is reduced feed intake (Pinton et al., 2008). Feeding pigs a diet naturally contaminated with DON at levels of 2 ppm decreased feed intake by 7.6% compared to pigs fed a diet containing 0 ppm DON (House et al., 2002). Although diets containing 1 or 2 ppm DON did not decrease average daily gain or feed efficiency, the number of

days for gilts to reach 110 kg increased by 14.1 days (House et al. 2002). Increasing the number of days to slaughter would create challenges to producers by affecting animal uniformity and due to increased labor and feed costs. In contrast, DON concentrations up to 8 ppm did not affect poultry productivity (Hamilton et al., 1985a, b). These findings were in agreement with work done by Awad et al. (2004) that showed broilers fed a diet containing 10 ppm DON had no reduction in growth performance, although there was a decrease in the rate of nutrient transfer in the intestine.

Ruminant species are less affected by DON than monogastric species. For example, dairy cows fed diets containing DON at 66 ppm for 5 days did not show a reduction in milk production or show signs of illness (Cote et al. 1986). Similarly, exposure of dairy cows to DON concentrations of 6.4 ppm in feed for 6 weeks did not reduce milk production or generate signs of illness (Trenholm et al., 1985).

The difference in tolerance between different species may be due to the difference in toxin absorption rates. Pigs absorb DON rapidly, with plasma concentrations peaking within 30 minutes of ingestion (Prelusky et al., 1988). The majority of the ingested DON is absorbed in the proximal part of the small intestine before de-epoxidation takes place, leading to the more adverse effects in pigs (Danicke et al., 2004). Chickens have a lower rate of absorption into plasma and tissues and rapid clearance of the toxin (Prelusky et al., 1986). The intestinal microflora in poultry has the ability to convert DON into less toxic substrates (He et al., 1992; Lun et al., 1986, 1988).

Deoxynivalenol inhibits protein production by binding to eukaryotic ribosomes (Larsen et al., 2004). Protein synthesis is an essential function in all tissues, but tissues that have rapidly growing and dividing cells are very susceptible to the toxin (Larsen et al., 2004). Susceptible tissues and systems therefore include: the lining of the digestive tract, bone marrow, lymph nodes, spleen, liver, and immune system (Pestka et al., 2004). Pigs fed a diet naturally contaminated with DON at levels of 2.2 – 2.5 ppm for nine weeks altered both the global and specific immune responses and increased the total serum IgA concentration by 46.7% (Pinton et al., 2008). The ability of DON to alter the regulation of immunoglobulins was also reported by Pestka and Smolinski (2005) in a related study on rodents.

Experimental results of the toxicology of DON are difficult to relate to real-life situations because other related mycotoxins are usually present including other *Fusarium* toxins such as 3-ac-DON and 15-ac-DON (Charmley and Trenholm, 2000) (Figure 1.2). For example, pigs weighing 20 to 45 kg refused feed naturally contaminated with DON more frequently than feed spiked with equal concentrations of pure DON (Forsyth et al., 1977).

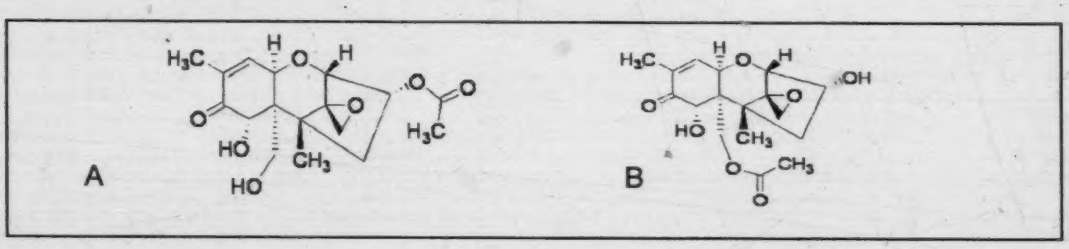

Figure 1.2. The chemical structure of 3-acetyl Deoxynivalenol (A) and 15-acetyl Deoxynivalenol (B).

Similar results were found with castrated male pigs (Prelusky et al., 1994). Diets containing naturally-infected corn with a DON content of 3 ppm decreased feed intake and weight gain, but the diet spiked with purified DON at equal concentration did not. These differences may be attributed to the presence of additional, naturally occurring toxins in the grain that affects DON toxicity.

1.1.3 Deoxynivalenol - Food Processing and Human Health

Once grain is infected with DON, little can be done to reverse the situation, resulting in food safety concerns. Operations such as sorting and cleaning of grain can reduce the amount of DON by removing contaminated material, shrunken kernels and fine pieces, but this does not destroy the toxin (Bullerman and Bianchini, 2007). In the milling process, any DON present can be redistributed or concentrated in different milling fractions, but none of the standard milling processes will eliminate or destroy DON (Bullerman and Bianchini, 2007).

DON is a very stable compound and is almost unaffected by storage or heat during processing such as baking, cooking or brewing (Scott, 1991). Regular bread production showed a range in DON reduction from 24 to 71%, while cookie and biscuit production showed a 35% reduction (Scott et al., 1983; Scott, 1984). Corn processed by canning has shown a 12% reduction in DON levels; however, DON is not reduced during baby food or dog food production (Wolf-Hall et al., 1999). Extrusion processing seems to have the highest impact

on DON concentrations. Moisture contents of 15 and 30%, temperatures of 150 and 180 °C, and addition of sodium metabisulphite during extrusion all achieved detoxification of greater than 95% (Cazzaniga et al., 2001).

In the malting and brewing industry, DON has been found in trace amounts in commercial beer (Scott, 1996). An even extremely low level of DON in malt has been related to 'gushing' in beer, the sudden over production of foam upon opening a bottle (Wolf-Hall, 2007). This is a serious quality defect that can lead to the loss of consumers.

Ingestion of DON in humans has been associated with gastroenteritis causing nausea emesis, diarrhea, anorexia, and gastrointestinal hemorrhaging as well as affecting immune function and protein inhibition (Pestka and Smolinski, 2005; Sergent et al., 2006). Chronic exposure to DON contaminated foods may negatively affect human health by altering the intestinal mucosa integrity and by inducing the mitogen-activated protein kinases implicated in inflammation (Sergent et al., 2006). *In vitro* studies done on human cells show that DON exposure could result in both stimulation (cytokine production) and impairment of the immune function, depending on dose and length of exposure (Pestka and Smolinski, 2005).

The capability of DON to act as a carcinogen has been difficult to determine. Deoxynivalenol was unable to act as a promoter or initiator of skin tumors in mice; however, it was capable of producing a moderate squamous hyperplasia (Lambert et al., 1995). In a mini-review by Ma and Guo (2008), 6 studies were described in the literature that related to severe contamination of

DON and high incidences of cancer in humans. In all 6 studies, food grain with high concentrations of DON was determined to be the causal agent for esophageal cancer. However, the studies failed to take into account the effects of other toxins that may have been present, and they failed to produce enough details to conclusively determine that DON was the toxin causing the cancer.

While the literature may not show conclusively the adverse effects of DON on human health, public opinion often outweighs any conclusive research. The fact that DON has such detrimental effects on pigs and pigs are used to model human intestine function, leads to the conclusion that DON will also adversely affect human health (Nejdfors et al., 2000).

1.2 Economic Impact of DON

When mycotoxosis causes animal death, it is easy to define the economic impact. However, consequences of DON contamination are more likely to cause lower productivity, affect immune function, cause reproduction failures, and other outcomes that are hard to measure. Because of near zero tolerance for DON, grain buyers and processors tend to avoid areas that have high incidence of the toxin, causing a devastating effect on grain producers when they are unable to sell their crops (Wu, 2007). Compared to other toxins that are generally more noxious, such as aflatoxin, far fewer nations have legislated acceptable limits for DON content in grains (Wu, 2007). Economic losses from DON contamination in the United States are higher due to the inability to meet internal standards set by

the US Food and Drug Administration (FDA) rather than loss from export markets (Wu, 2007).

Exact figures for economic losses resulting from DON contamination may never be accurately determined, because little published data are available. The 1996 *Fusarium* outbreak in Ontario resulted in estimated direct losses of over $100 million Canadian dollars (Schaafsma, 2000). In a review for the Food and Agriculture Organization of the United Nations (FAO), Bhat and Miller (1991) report that economic losses due to DON can be contributed to the obvious losses of food and feed, in addition to "losses caused by lower productivity; losses of valuable foreign exchange earnings; costs incurred by inspection, sampling and analysis before and after shipments; losses attributable to compensation paid in case of claims; farmer subsidies to cover production losses; research, training and extension program costs and costs of detoxification". It is clear that the economic impacts of DON contamination can be staggering.

1.3 Limits for DON Content

With the difficulty in removing DON from grain and the ability of the DON toxin to persist during processing and treatment, the need for detection of the toxin to prevent it from entering into the food and feed market is imperative. Many countries have regulated the amount of toxins in grain products at maximum tolerable levels and these are changing and even decreasing as the

technology of detecting mycotoxins advances. Malting companies have established a maximum level of 0.5 ppm DON in barley to be used for malting (McMullen et al., 1997), although some companies have instituted a zero tolerance for DON. Canadian guidelines for DON levels are 5 ppm on a dry matter basis for total feed intake for beef cattle, sheep and poultry; but only 1 ppm for swine, dairy cattle and horses (Manness, 2002). The limits for DON proposed by the EU Commission (Codex Alimentarius Commission FAO/WHO, 2003) for various cereal and cereal products are shown in Table 1.1. The maximum level of DON for various nations is shown in Figure 1.3 (Wu, 2007).

Table 1.1. Limits for deoxynivalenol content proposed by the EU Commission for various cereals and cereal products.

Cereals and Cereal Products	Limit of Deoxynivalenol Content (ppm)
Unprocessed durum wheat and corn	2
Other unprocessed cereals	1.5
Wholemeal wheat flour, bran and pasta (dry)	0.75
Cleaned cereal for direct humanconsumption and all derived products	0.50
Maize based breakfast cereals and snacks	0.50
Cereal for infants and ingredients used in manufacture thereof	0.10

Table 1.2. Maximum tolerable levels for DON in animal feeds in different nations.

Nation	Animal feed commodity	DON toxin standard (ppm)
Canada	Cattle/poultry feed	5
	Pigs/young calves/ lactating dairy feed	1
Austria	Breeding pig feed	0.05
	Other pig feed	0.5
	Cattle/breeding poultry feed	1
	Other poultry feed	1.5
Cuba	All feed	0.3
Cyprus	Pig feed	1
	Poultry / cattle feed	5
	All other feed	3
Iran	Cattle/sheep/goat feed	5
	Calf/lamb/kid feed	1
Japan	All feed	1
Lithuania	Pig feed	1
Romania	All feed	5
Slovenia	Pig and poultry feed	0.4
Ukraine	All feed	1
United States	Cattle/poultry feed	10
	Pig feed	5
	All other feed	5

Sources: (CAST, 2003), (FAO, 2004), (Wu, 2007)

1.4 Detection of Deoxynivalenol

Mycotoxin identification and quantification is required so that contamination can be managed effectively. The need to comply with regulations regarding toxin content has increased the interest in developing rapid and accurate analytical methods for field and commercial use. The toxins themselves are not visible to the naked eye, and analytical tests for fungi or mold spore counts are of little or no value due to the inconsistency of toxin production by the fungus (Whitney, 2004).

Current methodologies for determining DON in grain include: High Performance Liquid Chromatography (HPLC), Gas Chromatography (GC), Thin Layer Chromatography (TLC) and Enzyme-Linked Immunosorbent Assays (ELISA). For analysis of DON, GC and HPLC are commonly applied due to their high sensitivity and specificity (Scott, 1993; Gilbert, 1993). In most cases, GC analysis is used for determination of DON (Kotal et al., 1999) with 75% of the studies on DON using GC for DON determination and only 25% using HPLC (Scott et al., 1990). Gas chromatography provides both selectivity and sensitivity for DON quantification by using high-resolution columns together with optimized detection (Kotal et al, 1999). With proper calibration, GC can be used to detect many different toxins simultaneously with good separation between the peaks.

However, each toxin must be calibrated separately for accurate results. This requires preparing samples of known concentrations using purified toxin and using these samples to create a calibration curve for GC. Using GC analysis can be restrictive because it is labor and time intensive and requires highly skilled operators.

The focus has been to develop rapid tests for mycotoxin analysis, to accommodate the time constraints of industry. Commercial ELISA tests now have total test times of 20 to 60 minutes (Schneider et al., 2004); which is a quick and simple methodology compared to the GC test. The ELISA test is cheaper than GC or HPLC methods, but rapid tests in general are commonly considered as less accurate and unreliable (Schneider et al., 2004).

Comparison of GC and ELISA methodology shows a strong correlation. Using corn and wheat samples with a DON concentration ranging from 0 to 40ppm, the correlation between the two methodologies resulted in a $R^2 = 0.972$ (Sinha et al., 1996). When only the lower concentrations of DON were used, the R^2 dropped to 0.835 for corn under 5 ppm and $R^2 = 0.582$ for wheat samples below 2 ppm. This was due to the ELISA values being generally higher than the GC values. Other *Fusarium* toxins present, particularly 15-Ac-DON, were cross-reacting with the ELISA test causing inaccurate higher readings.

One feature of the ELISA test that must be carefully considered is test specificity and cross-reactivity. Most immunoassays for trichothecenes have moderate or strong cross-reactivity with closely related analogues (Schneider et al., 2004). Antibodies for DON can strongly cross-react with 3-AcDON or 15-

AcDON or with both (Schneider et al., 2004). Among the different ELISA kits, cross reactivity seems to be a common problem, and is difficult to quantify. The test specificity should be identified with any ELISA test as it is critical to ensure correct data interpretation.

The challenge is to be able to detect the toxin at the low concentrations legislated by industry and governments, while maintaining error terms low enough to be accurate. The limits of detection for DON analysis can be complicated. While most equipment manufacturers can give you the detection limits for their equipment or methodologies, real limits can only be determined in your lab, using your equipment, chemicals and technicians. Despite the utilization of these sophisticated and expensive procedures, agreement between laboratories is invariably poor, even when identical samples are analyzed (Coker, 1984). The precision and accuracy of analytical methods for mycotoxins in cereal grains is usually overshadowed by sampling errors (Scott, 1990).

1.4.1 Sampling

The most critical part of any analytical procedure for mycotoxins is sampling the commodity in a truly representative manner. Correct analytical results cannot be obtained without representative sampling (Larsen et al., 2004). This is especially true with mycotoxins because it is often distributed very heterogeneously throughout the sample in so-called "hot-spots" (Larsen et al., 2004). The variation in mycotoxin infection can lead to single kernels being

highly infected, while the majority of the sample can be clean. Proper sampling techniques have even been legislated in Europe by the Commission Directive 2002/26/EC (European Commission, 2002c). However, they do allow for some flexibility in the legislation that would allow different sampling techniques that may work better with processed products. Paragraph 4.4 of this Directive clearly says: "If it is not possible to carry out the method of sampling described above because of the commercial consequences resulting from damage to the lot (because of packaging forms, means of transport, etc.) an alternative method of sampling may be applied provided that it is as representative as possible and is fully described and documented". With large lots of grain, representative sampling would require sub-sampling the lots when loading or unloading, blending the sub-samples and further sampling for analysis. If the grain were to be ground, adequate mixing after grinding would also be necessary.

Published studies of aflatoxins in peanuts have shown that only 6% of the total testing error is due to the analytical method, while 94% is due to sampling and sub-sampling problems (Whitaker, 2003). Producers and handlers of grain are strongly advised to employ well-validated protocols in getting representative samples of grain for mycotoxin testing. This would ensure that the most accurate results are obtained. It is important to note that if one toxin is identified in a sample, the chances are high that other toxins are present.

1.5 Near Infrared Reflectance Spectroscopy (NIRS)

Near infrared reflectance spectroscopy has been utilized since the 1960's to predict quality characteristics and it has evolved into a useful tool for many industries world wide including pharmaceutical and agriculture. In the agriculture industry, NIRS is used in grain elevators and terminals to predict quality characteristics like protein and moisture. It has expanded into other areas of the industry and is well established in the analysis of agricultural products, especially forages (Dardenne et al., 1991; Shenk and Westerhaus, 1994), cereals (Oatway and Helm, 1999) and feedstuffs (Dardenne et al., 1993; Shenk et al., 1977).

Near infrared reflectance spectroscopy is a rapid method based on the absorption of electromagnetic radiation from approximately 780 to 2500 nm (Huang et al., 2008). Near infrared spectra are comprised of broad bands from overtones and combinations of C-H, N-H and O-H chemical bonds (Osborne, 2000). Depending on the chemical bonds present in the sample, the radiation will be absorbed at different rates and a unique spectra scan of the sample will be produced that can then be used in the development of calibration equations.

The NIRS is a secondary method of analysis in that primary, or wet chemistry analysis, is needed to calibrate the instrument before it can be used. NIRS calibration involves chemistry, physics and statistics to find a relationship between wet chemistry analysis and the absorption scan of a sample. This relationship defines a calibration equation that quickly and reproducibly predicts chemical characteristics for samples.

Different wavelengths in the near infrared spectrum correlate to distinct chemical structures. The pharmaceutical industry uses this principle to monitor the purity of different chemicals used in medicines and determining proper mixing of batches. Agricultural products are much more complex in that they do not contain a single chemical structure, but include a mixture of proteins, fiber, starch and other quality components that may be specific to each product. Using purified samples, certain wavelengths that are specific for these components can be identified. For example, work reported by Williams and Norris (2001) shows that that protein absorbs near infrared light at 36 different wavelengths and starch at 28 wavelengths. It is also important to note that absorption bands are not always isolated and bands for protein can overlap with each other as well as absorption bands for starch (Williams and Norris, 2001). To date there has been no studies found in the literature reporting work done on identifying the near infrared absorption spectra for DON, intended for developing NIRS calibrations.

The error in NIRS calibration is complicated. Generally, it can be categorized as sampling error, wet chemistry or reference error and NIRS calibration error. Sampling error is caused by a lack of homogeneity within the sample being tested. As mentioned earlier, sampling error become the primary concern when dealing with mycotoxins, due to the variable infection and production of toxins within the grain kernels. NIRS calibration error can be a combination of spectral measurement errors, lack of intrinsic correlation between spectral and wet chemistry data and data treatment and mathematical errors. In order to obtain a NIRS calibration with the capability of measuring DON at the

legislative limits, it is imperative to minimize as many sources of error as possible.

1.5.1 NIRS and DON

There is very little in the literature pertaining to the detection of mycotoxins using NIRS and those available have produced variable results. Many of the studies involve the sorting of *Fusarium* damaged kernels, but not determination of the toxin itself.

The NIRS has been shown to be useful for optical sorting of scab damaged kernels in wheat. Using the 500 to 550 nm range in the visible region, wheat samples were classified according to scab damage with 94% accuracy (Delwiche et al., 2005a). The same study showed similar results (97% classification accuracy) using 1150 and 1250 nm in the near infrared region. For the study, researchers used visually identified scab damaged and normal kernels of wheat for the classification; however, there was no mention of determining the concentration of toxin on the kernels. Even with non-scab damaged kernels, there could still be toxin present and this was not discussed. In another project by Delwiche et al. (2005b), visual measurements on the proportion of *Fusarium*-damaged kernels were collected on incoming and sorted seed as were DON concentration measurements using ELISA. Results of this project indicated that the fraction of DON content in the sorted wheat to that in the unsorted wheat ranged from 18 to 112%, with an average of 51%. The wide range of results

could be due to kernels showing no visual signs of infection but having high DON concentrations.

Similar results were found when using NIRS to sort corn contaminated with mycotoxins. For this study Wicklow et al. (2007) visually sorted and grouped corn kernels into four symptom categories: asymptomatic, showing 25% to 50% discoloration, showing over 75% discoloration, and discolored kernels. Using wavelengths at 500 and 1200 nm they were able to correctly classify 87% of the kernels infected with aflatoxin. However, they reported that some kernels with only minor symptoms of discoloration could have very high aflatoxin concentrations and this was one of the causes of the higher than expected errors. They also stated that "differences in pericarp thickness, kernel hardness and the presence of carotenoids could influence symptom expression and the ability of NIRS to detect fungal damaged endosperm".

Dowell et al. (1998) used NIRS measurements to detect scab and estimate DON levels in single kernels of wheat. In this study, only visibly damaged kernels were used in their prediction model. The R^2 of their model was 0.64 and the standard error of prediction (SEP) was too high for industry use at 44 ppm.

Ruan et al. (2002) looked at using a neural network and NIRS to predict actual DON concentration. Their NIRS calibration produced an R^2 value of 0.93 and a SEP of 3.1 ppm. This shows that NIRS has the potential to predict DON, although the SEP is to high to provide accurate results at the legislated limits for DON. Their results showed that the near infrared region and visible region were

sensitive to the concentration range of components such as moisture, protein and colorants and hypothesized that these could be highly correlated to DON in barley. They concluded that the DON level in barley could be determined through the spectra of the visible region. This is in disagreement with the findings of Wicklow et al. (2007), which suggested that caratenoids interfered with the NIRS classification of DON.

Another study by Pettersson and Aberg (2003) developed a NIRS calibration for DON concentration. Using near infrared transmittance (NIT) and a Foss Tecator Infratec™ (wavelength range of 570 to 1100 nm), they developed a calibration using whole grain samples from Sweden and Norway. For this study, DON infected samples were diluted in different series to produce their calibration set. Their calibration model utilized 11 to 13 factors to predict DON and they indicated that some of these factors may have come from other compounds or effects related to DON and produced by *F. culmorum*. Their best equation had an R^2 value of 0.984 and a standard error of cross validation (SECV) of 381 ppb. When this calibration was used to predict a set of samples from Austria the slope for cross validation was much higher. The Austrian material was more visibly infected having a higher concentration of pink and shriveled kernels and an increased absorption in the visible region. When these samples were added to the calibration, the R^2 values decreased to 0.90 and the SECV values increased to 1234 ppb. In their final conclusions, Pettersson and Aberg (2003) stated that the component in the model not directly related to DON concentration needed to be minimized or eliminated.

As discussed earlier, researchers have had conflicting results when relating disease incidence or severity to mycotoxin concentrations. This variability in disease expression and toxin formation could be causing the difficulties in the calibration of NIRS. With the relationship between fungal infection and toxin formation not consistent, any calibration that was focusing on the presence of the fungus is flawed. Several of the above researchers came to the conclusion that components not related to the DON absorption should be eliminated to improve the ability of NIRS to predict DON. By using the entire wavelength range, calibrations are influenced by color and physical characteristics leading to decreased predictability of DON and higher errors.

1.6 Current State of Knowledge

There have been very few studies investigating the ability of NIRS to predict DON levels in grain. Calibrations have been reported with correlations between 0.64 to 0.98, showing there is a definite potential to use NIRS for DON determination. However, these experiments also indicate that factors relating to *Fusarium* infection, such as shrunken and discolored kernels, that are unrelated to DON content could be negatively affecting calibration development.

1.6.1 Gaps in Knowledge

Previous NIRS calibrations have focused on detecting the effects of *Fusarium* on the seed such as shrunken kernels or discoloration. However, studies have been inconsistent in terms of relating DON levels with fungal infection and this is causing inaccuracies within the previously developed models. There needs to be a precise method developed that will be uninfluenced by the physical characteristics of *Fusarium* infected grain and concentrate only on the presence of the toxin.

The use of specific wavelengths to develop a calibration model specifically for DON has not been investigated so far. Currently, there is no published information on the absorption characteristics of DON, and the specific absorption spectrum of DON has yet to be determined. By determining the regions that are specific for the toxin and limiting our calibration model to these wavelengths, the calibration should be more precise and specific for DON concentration.

1.7 Scope of Thesis

The aim of the present thesis was to determine the ability of NIRS to precisely detect the presence of DON. Since previous works in this area have shown the potential to develop this technology for detecting toxins, this research will look at more novel approaches to refining the technology to improve its accuracy. Since there has been no work done in isolating the absorption

spectra of DON, this will be the initial focus (Chapter 2). Using purified DON, the primary NIRS absorption peaks will be isolated and recorded. Artificial standards using pure DON and wheat flour will be prepared and used to test the new calibration model.

The specific wavelengths isolated for DON characterization will be used to develop a NIRS calibration for DON using naturally infected wheat and barley (Chapter 3). Using specific wavelengths should eliminate any conflicting information and lead to a calibration equation that will truly represent DON. Since GC analysis produce very accurate results, with low detection limits, it will be chosen as the method to develop the NIRS model. The calibration will be tested against a full spectrum calibration model to test the differences between wavelength range and prediction capability.

1.8 Summary

The agricultural industry worldwide has been negatively impacted by fungal contamination. The effects of *Fusarium* infection on crops are two fold. Not only does it result in decreased yield, shrunken kernels and other physiological effects in the plant, but the fungus is responsible for the production of DON that effects the final quality of the grain and in severe cases renders the grain unusable. While the presence of the fungus can indicate a potential hazard, only the detection of toxin is useful.

To counteract this hazard, a quick, precise methodology to detect and

quantify the amount of toxin present is required so that producers and industry can control the impact. Current methodologies require skilled operators and require too much time for 'real-time' operations. Near infrared spectroscopy is one method that can bridge this gap and provide the analysis needed. This technology is familiar to the agriculture industry and analyzes quality characteristics for a wide-range of products in feed and food manufacturing. However, in order to use this technology there needs to be calibration equation developed using traditional procedures for quantifying DON.

References

Abramson, D., J.T. Mills, R.R. Marquardt and A.A. Froehlich. 1997. Mycotoxins in fugal contaminated samples of animal feed from western Canada, 1982 – 1994. Can. J. Vet. Res. 61: 49-52.

Awad, W.A., J. Bohm, E. Razzazi-Fazeli, H.W. Hulan, and J. Zentek. 2004. Effects of deoxynivalenol on general performance and electrophysiological properties of intestinal mucosa of broiler chickens. Poult. Sci. 83: 1964–1972.

Bai, G.H., R. Plattner, A. Desjardins and F. Kolb. 2001. Resistance to *Fusarium* head blight and deoxynivalenol accumulation in wheat. Plant Breed. 120:1–6.

Bhat, R.V. and J.D. Miller. 1991. Mycotoxins and food supply. J. Food, Nutr. and Agriculture. Available at http://www.fao.org/docrep/U3550t/u3550t0e.htm (accessed March, 2008).

Bennett, J.W., and M. Klich. 2003. Mycotoxins. Clin. Microbiol. Rev. 16: 497-516.

Biner, E.M., L.M. Tan, L.J. Chin, J. Handl and J. Richard. 2007. Worldwide occurrence of mycotoxins in commodities, feeds and feed ingredients. Anim. Feed Sci. Tech. 137: 265-282.

Bullerman, L.B. and A. Bianchini. 2007. Stability of mycotoxins during food processing. Intern. J. Food Micro. 119: 140–146.

CAST, 2003. Mycotoxins, economic and health risks. Task Force Report No. 116. Council for Agricultural Science and Technology, Ames, IA.

Cassaniga, D., J.C. Basilico, R.J. Gonzalez, R.L Torres, and D.M. de Greef. 2001. Mycotoxins inactivation by extrusion cooking of corn flour. Lett. Appl. Microbiol. 33: 144 – 147.

Charmley, L.L., and H.L.Trenholm. 2000. A Review of Current Literature on Mycotoxins and Their Regulations. (Unpublished review for Canadian Food Inspection Agency, Government of Canada).

Charmley, L.L., H.L. Trenholm, D.B. Prelusky and A. Rosenberg. 1995. Economic losses and decontamination. Mat. Toxins 3: 199-203.

Clear, R.M. and S.K. Patrick. 2000. *Fusarium* head blight pathogens isolated from *Fusarium*-damaged kernels of wheat in western Canada, 1993 to 1998. Can. J. Plant Path. 22:51-60.

Codex Alimentarius Commission FAO/WHO. 2003. Codex Committee on Food Additives and Contaminants, 35th Session. Discussion paper on deoxynivalenol. Arusha, Tanzania, 17–23 March 2003.

Cochrane, B. 2007. *Fusarium* levels lower than usual - reduced risk of mycotoxins. The PigSite. http://www.thepigsite.com/swinenews/15932/surveys-show-low-levels-of-*Fusarium*-head-blight-infection-on-the-prairies-in-2007. (accessed March, 2008).

Coker, R. D. 1984. In Analysis of Food Contaminants (J Gilbert, editor), Elsevier Applied Science Publishers, London and New York, pp 207-263.

Commission Directive (EC 1998) No 1998/53/EC of 16 July 1998 laying down the sampling methods and the methods of analysis for the official control of the levels for certain contaminants in foodstuffs. Official FAO Journal 201: 93-101.

Commission Directive 2002/26/EC of 13 March 2002 laying down the sampling methods and the methods of analysis for the official control of the levels of ochratoxin A in foodstuffs. Official FAO Journal 75: 38-43.

Commission Directive 2002/27/EC of 13 March 2002 amending Directive 98/53/EC laying down the sampling methods and the methods of analysis for the official control of the levels for certain contaminants in foodstuffs. Official FAO Journal 75: 44-45.

Commission Regulation (EC 2002b). No 472/2002 of 12 March 2002 amending regulation (EC) no. 466/2001 setting maximum levels for certain contaminants in foodstuffs. Official FAO Journal 77: 1-13.

Cote, L.M., A.M. Dahlem, T. Yoshizawa, S.P. Swanson, W.B. Buck. 1986. Excretion of deoxynivalenol and its metabolite in milk, urine, and faeces of lactating dairy cows. J. Dairy Sci. 69: 2416–2423.

Cromey, M.G., S.C. Shorter, D.R. Lauren, K.I. Sinclair. 2002. Cultivar and crop management influences on *Fusarium* head blight and mycotoxins in spring wheat (*Triticum aestivum*) in New Zealand. N.Z. J. Crop Hort. Sci. 30:235–247.

Danicke, S., H. Valenta, S. Doll, M. Ganter, and G. Flachowsky. 2004. On the effectiveness of a detoxifying agent in preventing fusario-toxicosis in fattening pigs. Anim. Feed Sci. Technol. 114: 141-157.

Dardenne, P., J. Andrieu, Y. Barriere, R. Biston, C. Demarquilly, N. Femenias, M. Lila, M. Maupetit, F. Riviere and T. Ronsin. 1993. Composition and nutritive value of whole maize plants few fresh to sheep. 2. Prediction of the in vivo organic matter digestibility. Ann. Zootech. 43: 251-270.

Dardenne, P., G. Sinnaeve, R. Biston, and P. Lecomte. 1991. Evauation of NIT for predicting fresh forage quality. In: Murray, I., Cowe I.A. (eds.), Making Light Work: Advances in NIR Spectroscopy, VCH. Weinheim, pp. 269-271.

Delwiche, S.R., C.S. Gaines. 2005a. Wavelength selection for monochromatic and bichromatic sorting of *Fusarium*-damaged wheat. Appl. Eng. Agric. 21: 681-688.

Delwiche, S. R., T.C. Pearson and D.L. Brabec. 2005b. High-speed optical sorting of soft wheat for reduction of deoxynivalenol. Plant Dis. 89:1214-1219

Desjardins, A.E. 2006. *Fusarium* Mycotoxins: Chemistry, Genetics, and Biology. APS Press, St. Paul, Minnesota, USA.

Desjardins, A.E., and R.H. Proctor. 2001. Biochemistry and genetics of *Fusarium* toxins. (pp. 50-69) Fusarium: Paul E. Nelson Memorial Symposium. B.A. Summerell, J.F. Leslie, D. Backhouse, W.L. Bryden and L.W. Burgess. American Phytopathological Society Press, St Paul, MN. St. Paul, Minnesota, USA.

Desjardins A.E., T.M. Hohn, S.P. McCormick. 1993. Trichothecene biosynthesis in *Fusarium* species: chemistry, genetics, and significance. Microbiol. Rev. 57: 595-604.

Dowell, F. E., M.S. Ram and L.M. Seitz. 1999. Predicting scab, vomitoxin, and ergosterol in single wheat kernels using near infrared spectroscopy. Cereal Chem. 76: 573–576.

D'Mello, J.P.F. and A.M.C. Macdonald. 1997. Mycotoxins. Anim. Feed Sci. Technol. 69: 155-166.

FAO (Food and Agriculture Organization), 2004. Worldwide Regulations for Mycotoxins in Food and Feed in 2003. FAO Press, Rome, ISBN 92-5-105162-3.

Fink-Gremmels, J. 1999. Mycotoxins: their implications for human and animal health. Vet. Quat. 21:115-120.

Fernandez, M. R., P.G. Pearse, G. Holzgang, and G.R. Hughes. 2002. *Fusarium* head blight in common and durum wheat in Saskatchewan in 2001. Can. Plant Disease Survey. 82: 36–38.

Forsyth, D.M., T. Yoshizawa, N. Morooka, and J.Tuite. 1977 Emetic and refusal activity of deoxynivalenol to swine. Appl. Environ. Microbiol., 34: 547–552.

Gilbert, J. 1993. Recent advances in analystical methods for mycotoxins. Food Add. Contam. 10: 37-48.

Gilbert, J., A. Tekauz, D.A. Kaminski, R. Kaethler, E. Mueller, and U. Kromer. 2002. 2001 *Fusarium* head blight survey of spring wheat in Manitoba. Can. Plant Disease Survey. 82: 44.

Hamilton, R.M., B.K. Thompson, H.L. Trenholm, P.S. Fiser,and R.Greenhalgh. 1985a. Effects of feeding white Leghorn hens diets that contain deoxynivalenol (vomitoxin)-contaminated wheat. Poult. Sci. 64: 1840–1852.

Hamilton, R.M., H.L. Trenholm, B.K. Thompson, and R. Greenhalgh. 1985b. The tolerance of White Leghorn and broiler chicks, and turkey poults to diets that contained deoxynivalenol (vomitoxin)-contaminated wheat. Poult. Sci. 64: 273–286.

He, P., L.G. Young, and C. Forsberg. 1992. Microbial transformation of deoxynivalenol (vomitoxin). Appl. Environ. Microbiol. 58: 3857–3863.

House, J.D., D. Abramson, G.H. Crow, and C.M. Nyachoti. 2002. Feed intake, growth and carcass parameters of swine consuming diets containing low levels of deoxynivalenol from naturally contaminated barley. Can. J. Anim. Sci. 82: 559-565.

Huang, H., H. Yu, H. Xu, Y. Ying. 2008. Near infrared spectroscopy for on/in-line monitoring of quality in foods and beverages: A review. Food Eng. 87:303–313.

Kotal, F., K. Holadova, J. Hajslova, J. Poustka, and Z. Radova. 1999. Determination of trichothecenes in cereals. Chromatogr. 830: 219-225.

Krska, R., E. Welzig and H. Boudra. 2007. Analysis of *Fusarium* toxins in feed. Anim. Feed Sci. Tech. 137: 241 – 264.

Lambert, L.A., F.A. Hines, and R.M. Eppley. 1995. Lack of initiation and promotion potential of deoxynivalenol for skin tumorigenesis in sencar mice. Food Chem. Toxicol. 33 (3): 217–222.

Larsen, J.C., J. Hunt, I. Perrin and P.Ruckenbauer. 2004. Workshop on trichothecenes with a focus on DON: summary report. Toxicol. Letters 153: 1–22.

Lauren D.R. and W.A. Smith. 2001. Stability of *Fusarium* mycotoxins nivalenol, deoxynivalenol and zearalenone in ground maize under typical cooking conditions. Food Addit. Contam. 18: 1011-1016.

Lun, A.K., E.T. Moran Jr., L.G. Young, and E.G. McMillan. 1988. Disappearance of deoxynivalenol from digesta progressing along the chicken's gastrointestinal tract after intubation with feed containing contaminated maize. Bull. Environ. Contam. Toxicol. 40: 317–324.

Lun, A.K., L.G. Young, E.T. Moran Jr., D.B. Hunter, and J.P. Rodriguez. 1986. Effects of feeding hens a high level of vomitoxin-contaminated maize on performance and tissue residues. Poult. Sci. 65: 1095–1099.

Ma, Y.Y. and H.W. Guo. 2008. Mini-review of studies on the carcinogenicity of deoxynivalenol. Environ. Toxicol. Pharmacol. 25: 1-9.

Manitoba Agriculture, Food and Rural Initiatives. 2007. Mycotoxins factsheet [online]. Available at http://www.gov.mb.ca/agriculture/foodsafety/processor/cfs02s70.html (accessed March, 2008).

Manness, G., T. Nicholson, and C. Nicolaou. 2002. Ethanol: made in Manitoba. Winnipeg, MB: Manitoba Energy Development Initiative.

McMullen, M., Jones, R., Gallenberg, D., 1997. Scab of wheat and barley: a reemerging disease of devastating impact. Plant Dis. 81, 1340–1348.

Miedaner, T., B. Schneider, and H.H. Geiger. 2003. Deoxynivalenol (DON) content and *Fusarium* head blight resistance in segregating populations of winter rye and winter wheat. Crop Sci. 43: 519–526.

Morooka N., N. Uratsuji, T. Yoshizawa, and H. Yamamoto. 1972 Studies on the toxic substances in barley infected with *Fusarium* spp. J. Food Hyg. Soc. Jpn. 13: 368-375.

Nejdfors, P., M. Ekelund, B. Jeppsson, B. Westrom. 2000. Mucosal in vitro permeability in the intestinal tact of the pig, the rat, and man. Scand. J. Gastroenterol. 35: 501–507.

Oatway, L.A. and J.H. Helm. 1999. The Use of NIRS to Determine Quality Characteristics in Whole Grain Barley. Poster Presentation: 16th American Barley Researchers Workshop, July 11 – 15, Idaho Falls, ID, USA.

Oldenburg, E., Valenta, H., Sator, Ch., 2000. Risiskoabschätzung und Vermeidungs-strategien bei der Futtermittelerzeugung. In: Dänicke, S., Oldenburg, E. (Eds.), Risikofaktoren für die *Fusarium* toxinbildung und Vermeidungsstrategien bei der Futtermittelerzeugung und Fütterung, Sonderheft 216, Landbaufoschung Völkenrode, pp. 5–34.

Osborne, B.G., 2000. Near infrared spectroscopy in food analysis. BRI Australia Ltd, North Ryde, Australia. Copyright _ 2000 Wiley, New York, (Chapter 1) pp. 1–14.

Pestka, J.J., and A.T. Smolinski. 2005. Deoxynivalenol: toxicology and potential
effects on humans. J. Toxicol. Environ. Health B Crit. Rev. 8: 39–69.

Pestka, J.J., H.R. Zhou, Y. Moon, Y.J. Chung,. 2004. Cellular and molecular mechanisms for immune modulation by deoxynivalenol and other trichothecenes: unravelling a paradox. Toxicol. Lett. 153: 61–73.

Pettersson, H. and L. Aberg. 2003. Near infrared spectroscopy for determination of mycotoxins in cereals. Food Control. 14: 229-232.

Pinton, P., F. Accensi, E. Beauchampa, A.M. Cossalter, P. Callu, F. Grosjean, I.P. Oswald. 2008. Ingestion of deoxynivalenol (DON) contaminated feed alters the pig vaccinal immune responses, Toxicol. Lett. (In Press).

Placinta C.M., J.P.F. D´Mello and A.M.C. Macdonald. 1999. A review of worldwide contamination of cereal grains and animal feeds with *Fusarium* mycotoxins. Anim. Feed Sci. Technol. 78: 21-37.

Prelusky, D.B., R.G. Gerdes, K.L. Underhill, B.A. Rotter, P.Y. Jui, H.L. Trenholm. 1994. Effects of low-level dietary deoxynivalenol on haematological and clinical parameters of the pig. Nat. Toxins 2: 97–104.

Prelusky, D.B., K.E. Hartin, H.L.Trenholm and J.D. Miller. 1988. Pharmacokinetic fate of 15C-labeled deoxynivalenol in swine. Fundament. Appl. Toxicol. 10: 276-286.

Prelusky, D.B., R.M.G. Hamilton, H.L. Trenholm and J.D. Miller. 1986. Fundament. Appl. Toxicol. 7: 635 – 645.

Richard, J.L. 2007. Some major mycotoxins and their mycotoxicoses—An overview International Food Microbiol. 119: 3–10.

Rotter B.A., D. B. Prelusky, J.J. Pestka. 1996. Toxicology of deoxynivalenol vomitoxin. J. Toxicol. Environ. Health 48:1-34.

Ruan, R., Y. Li and P. Chen. 2002. Non-Destructive Determination of Deoxynivalenol Levels in Barley Using Near-Infrared Spectroscopy. Appl. Eng. Agric. 18(5): 549-553.

Schaafsma, A.W. 2000. Economic changes imposed by myctotoxins in food grains: case study of deoxynivalenol in winter wheat. Mycotoxins and food safety: Proceedings of an American Chem. Society Symp. Washington, DC. USA. August 21-23, 2000

Schneider, E., V. Curtui, C. Seidler, R. Dietrich, E. Uslebar and E. Martlbauer. 2004. Rapid methods for deoxynivalenol and other trichothecenes. Toxicol. Lett. 153: 133-121.

Scott, P.M. 1984. Effects of food processing on mycotoxins. J. Food Protect. 47(6): 489–499.

Scott, P.M. 1990. Mycotoxins, general referee reports. J. Assoc. Off. Anal. Chem. 73 (1): 98.

Scott, P.M.. 1991. Possibilities of reduction or elimination of mycotoxins present in cereal grains. In J. Chelkowski (Ed.), *Cereal Grain. Mycotoxins, fungi and quality in drying and storage* Amsterdam: Elsevier. pp. 529-572.

Scott, P.M. 1993. Gas chromatography of mycotoxins. In: Betina, V. (ed.), Chromatography of mycotoxins: Techniques and applications, Elsevier, Amsterdam, pp. 373-425.

Scott, P.M. 1996. Mycotoxins transmitted into beer from contaminated grains during brewing. J. of AOAC Intern. 79(4): 875–882.

Scott, P.M., S.R. Kanhere, P.Y. Lau, J.E. Dexter, and R. Greenhalgh. 1983. Effects of experimental flour milling and breadbaking on retention of deoxynivalenol (vomitoxin) in hard red spring wheat. Cereal Chem. 60: 421–424.

Scott, P.M., S.R. Kanhere, J.E. Dexter, P.W. Brennan, and H.L. Trenholm. 1984. Distribution of DON during the milling of naturally contaminated hard red spring wheat and its fate in baked products. Food Addit. Contam. 1(4): 313–323.

Sergent, T., M. Parys, S. Garsou, L. Pussemier, Y.J. Schneider and Y. Larondelle. 2006. Deoxynivalenol transport across human intestinal Caco-2 cells and its effects on cellular metabolism at realistic intestinal concentrations. Toxicol. Lett. 164: 167–176.

Shenk, J. S., M. O. Westerhaus. 1994. The application of near infrared relectance spectroscopy (NIRS) to forage analysis. In: Fashey, G. C., Jr. (Ed.), Forage Quality, Evaluation, and Utilization, Madison, WI, pp. 406 – 449.

Shenk, J.S., K. H. Norris, R. F. Barnes and G. W. Fissel. 1977. Forage and feedstuff analysis with an infrared reflectance spectro-computer system. Proc. 13th Int. Grassland Congress, Leipzig, German Democratic Republic, pp. 454 – 463.

Sinha, R.C. and M.E. Savard. 1996. Comparison of immunoassay and gas chromatography methods for the detection of the mycotoxin deoxynivalenol in grain samples. Can. J. Plant Pathol. 18:233-236.

Trenholm, H.L., B.K. Thompson, K.E. Hartin, R. Greenhalgh, A.J. McAllister. 1985. Ingestion of vomitoxin (deoxynivalenol)-contaminated wheat by nonlactating dairy cows. J. Dairy Sci. 68: 1000–1005.

Whitaker, T.B. 2003. Standardisation of mycotoxin sampling procedures: an urgent necessity. Food Control. 14:233-237.

Whitney, M. 2004. Effect of Mycotoxins in Swine. University of Minnesota Extension Service. Bulletin M1179, 2004.

Wicklow, D.T., T.C. Pearson and D.L. Brabec. 2007. NIR spectroscopy as a tool for optimizing sorting of white corn kernels contaminated with mycotoxins. Corn Dry Milling Conference Proceedings. May, 2007.

Williams, P. and K. Norris (Eds.) 2001. Near-Infrared Technology in the Agricultural and Food Industries, 2nd edition. American Association of Cereal Chemists, Inc., St. Paul, Minnesota, USA.

Wolf-Hall, C.E. 2007. Mold and mycotoxin problems encountered during malting and brewing. Intern. J. Food Micro. 119:89-94.

Wolf-Hall, C.E., M.A. Hanna, L.B. Bullerman. 1999. Stability of deoxynivalenol in heat treated foods. J. Food Protection 62: 962–964.

Wu, F. 2007. Measuring the economic impacts of *Fusarium* toxins in animal feeds. Anim. Feed Sci. Tech. 137 (2007) 363–374.

Zhou, W.-C., Kolb, F.L., Bai, G.-H., Domier, J.-B., 2002. Effect of individual Sumai 3 chromosomes on resistance to scab spread within spikes and deoxynivalenol accumulation within kernels in wheat. Hereditas 137:81–89.

Chapter 2

Isolation of Near Infrared Absorption Peaks for Deoxynivalenol

2.1 Introduction

The effect of *Fusarium* infection on agricultural crops is two fold. Not only does it decrease yield, produce shrunken kernels and other physiological effects in the plant, the fungus is responsible for the production of mycotoxins that affect the final quality of the grain and in severe cases renders the grain unusable. One of the most frequently detected *Fusarium* toxins in cereal crops is DON, which is a type B trichothecene (Rotter et al., 1996). DON is a very stable compound and is almost unaffected by storage or by heat during processing such as baking, cooking or brewing (Scott, 1991). All animal species evaluated in the literature have shown adverse effects from DON ingestion or inhalation; swine are the most sensitive to the toxin while ruminant animals are themost resistant (Prelusky et al., 1994).

The most limiting factor in adequately managing DON infected grain is a mean to detect the toxin quickly and economically, and therefore limit the amount entering into the food chain. Near Infrared Reflectance Spectroscopy (NIRS) is used throughout the grain, feed and food industries as it can screen large numbers of samples quickly and economically. As a result, there is increasing interest among these industries in the use of NIRS for mycotoxin determination.

Very few studies in the literature have looked at NIRS's capability of detecting mycotoxins. Wicklow et al. (2007) utilized NIRS to optimize sorting of corn kernels contaminated with mycotoxins. By using discriminate analysis and 2 wavelengths (500 and 1200 nm), 87% of the kernels infected with aflatoxin were correctly classified. They also found that kernels with only minor symptoms of discoloration could have very high contamination levels of aflatoxin and this was one of the causes of the higher than expected errors.

Several studies have looked at using NIRS specifically for the determination of DON with varying degrees of success. Utilizing naturally infected DON samples and diluting them in different series to produce a calibration set, Petterson and Aberg (2003) developed an equation using near infrared transmittance (NIT). The best equation developed had an R^2 value of 0.984 and a standard error of cross validation (SECV) of 381 ppb. However, when this equation was used to predict a set of samples from outside their calibration set that had a higher concentration of pink, shriveled kernels and thus an increased absorption in the visible region, the R^2 values decreased to 0.90 and the SECV values increased to 1234 ppb. They concluded that other components in the model not directly related to DON concentration had to be eliminated to produce better results. Ruan et al. (2002) also concluded that NIRS calibration was highly correlated to the colorants caused by *Fusarium* damage. The weakness of all of these studies is the assumption that discoloration, visibly damaged kernels or other physical characteristics of the grain caused by fungal infection is highly correlated to DON production.

While the visual identification of the fungus can indicate a potential hazard, only the detection of toxin is useful. Even if the fungus is present, the toxin is not always produced (Desjardins et al., 1993; Fink-Gremmels, 1999). Although there have been many studies on relating disease incidence or severity of *Fusarium* infection to mycotoxin concentrations, there have been conflicting results. Most studies report a positive correlation (Bai et al., 2001; Cromey et al., 2002; Miedaner et al., 2003) but others found no significant relationship between infection and toxin production (Zhou et al., 2002).

In the development of NIRS calibrations, researchers have been able to isolate areas of the spectrum that are indicative of specific componenets such as protein, fiber, oil and starch (Williams and Norris, 2001). This technique can be applied to isolate the absorbance spectrum of DON, minimize the effects of *Fusarium* damage on the calibration model and truly develop a calibration for DON content. Therefore, the objective of this study was to isolate the regions in the NIRS spectrum that are specifically related to DON content, test these wavelengths using calibration standards of known DON concentration and establish a baseline calibration for DON content that should minimize or eliminate the influence of color and physical abnormalities on the calibration.

2.2 Materials and Methods

2.2.1 NIRS Scanning

All the samples for the study were scanned using a FOSS

NIRSystems 6500 (Foss North America, Eden Prairie, MN, USA) spectrophotometer equipped with a transport module. A quarter size sample cup (46 X 46 mm) was used with a total surface area of 21.16 cm^2 for spectra collection. Approximately 5 g of each sample was poured into the sample cup, excess sample was scraped off to distribute the sample evenly and the lid pressed onto the back. The instrument recorded 32 scans per sample that were averaged to produce 1 spectrum (Shenk and Westerhaus, 1992). Data for each spectrum was collected from 400 to 2500 nm at 2 nm intervals using the integrated software WINISI II (FOSS NIRSystems Inc., Silver Spring, MD, USA) for 1050 absorption readings per sample. Absorption was recorded as log of 1/reflection (Shenk and Westerhaus, 1992).

2.2.2 Scanning of Pure DON

Pure DON samples (5 mg each) purchased from Sigma-Aldrich (Sigma-Aldrich Inc, St. Louis, MO, USA) were used to determine the specific absorption peaks of the toxin. However, not enough pure DON was available to fill a sample cup and obtain absorption spectra directly. Therefore, to obtain the spectra, an empty small ring cup with a black rubber back in place was scanned in the instrument. Five mg (1 vial) of DON was then placed into the cup, and it was scanned again in the instrument. The same sample cup and rubber backing, oriented in the same direction, was used for both scans to minimize any variation caused by the cup and backing itself. The amount of DON in the

sample cup was visible to the naked eye, but covered less than 5% of the surface area of the cup and the rubber back was clearly visible through the DON.

Subtracting one scan from the other using the GRAPHICS option in WINISI II removed the absorption peaks caused by the backing. The resulting difference spectrum displayed in the GRAPHICS option allowed visual identification of the absorption peaks of interest.

2.2.3 Calibration Set Development

To develop a set of samples for calibration, pure DON purchased from Sigma-Aldrich (Sigma-Aldrich Inc, St. Louis, MO, USA) was mixed with commercial white wheat flour in a series of dilutions to develop calibration standards of known concentration. To create the initial dilution standard of 2000 ppm, 8 vials of DON (5 mg each, 40 mg total) were mixed with 20 g of flour by placing into a plastic test tube with a screw top and mixing for 3 min using a test tube shaker. Fifteen g were taken from this initial dilution and added to 15 g of pure flour and mixed (as described above) to produce the 1000 ppm standard. Twenty grams of the 1000 ppm standard were mixed with 20 g of pure flour to produce the 500 ppm standard and this process was repeated to produce six additional standards with DON concentrations calculated to be 222.2, 111.0, 55.5, 27.8, 13.9, and 6.9 ppm, respectively. Since DON concentration was determined in the samples by calculation and not a reference method, the entire dilution series was repeated, producing a second set of dilution samples to test

the consistency of the mixing.

From each standard, 3 samples of approximately 5 g each were sub-sampled for scanning. Sub-samples from both dilution series along with 6 samples of the pure flour (0 ppm) yielded 60 standards with a level of DON concentration from 2000 ppm to 0 ppm. All 60 standards were scanned and the absorption spectra recorded as outlined above.

While the samples with DON contents from 2000 to 55.5 ppm were crucial in developing the calibration standards and ensuring adequate mixing, only the dilution standards with DON content of 27.8 ppm and less were used to develop the NIRS calibration. The target area for the calibration was in the 0 to 5 ppm range, which covered most of the current regulations for DON inclusion in animal feed and human food markets. Inclusion of samples up to 27.8 ppm extended this span 10 times to give the range needed to develop an equation, while eliminating samples above this level was expected to decrease the errors associated with variation at the higher concentrations, and errors associated with non linearity of the concentration curve. The resulting calibration would increase the accuracy of the calibration equation in the low concentration range for DON.

2.2.4 Development of the Calibration Equations

Spectral information was subjected to mathematical procedures using WINISI II calibration software. Calibration models were developed over the range of 400 to 2498 nm, as well as modified wavelength ranges to compare

results. The best combination found after evaluating many different derivatives and regression algorithms was a modified partial least squares (MPLS) math treatment (Shenk and Westerhaus, 1992) using the first derivative, calculated over a 4 point (8 nm) gap and a 4 point (8 nm) smooth (1,4,4,1 software setup). The software was setup for 8 cross validation groups and all calibrations developed used the same math treatment and setup.

Since all the dilution samples were developed using flour of the same composition, Principle Component Analysis (PCA) was applied to perform a preliminary study of the structure of the data set. PCA preferentially maximizes the differences in DON concentration using spectral differences to create variation between different materials (Williams and Norris, 2001). The use of principle components enhances the existing spectral differences between materials, an important feature due to the extremely low concentrations of toxins that were being detected.

2.3 Results and Discussion

2.3.1 Pure DON Calibration Analysis

Scanning the empty sample cup and the same cup with the 5 mg of DON added resulted in obvious differences in the spectral scan. As shown in Figure 2.1 the empty sample cup shows a relatively flat, non-descript absorption scan indicative of the black rubber. Black rubber was chosen for its lack of significant peaks in the near infrared region so that it would not cause interference with the

detection of DON. In comparison, the scan of the sample cup containing the DON (Figure 2.2) shows many peaks that can be utilized for calibration.

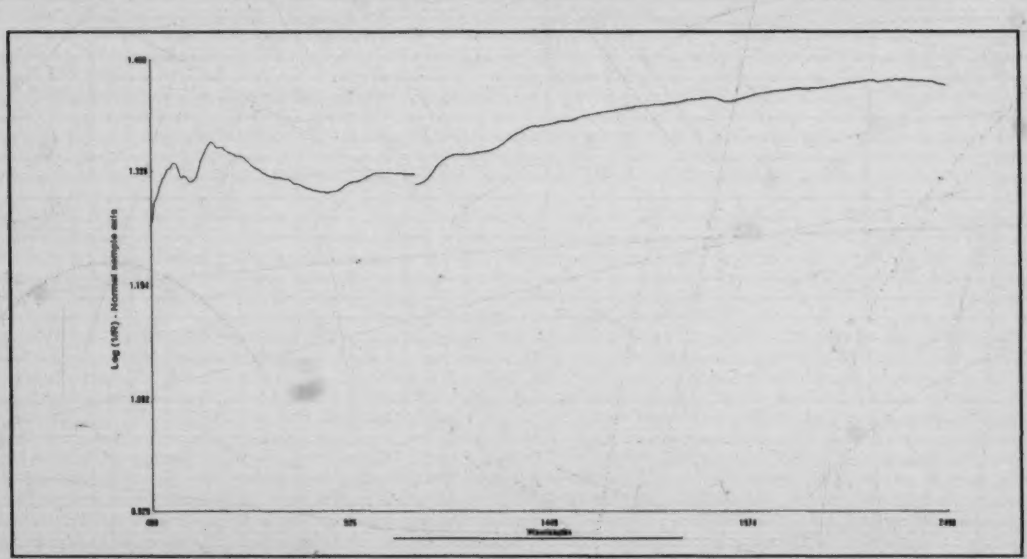

Figure 2.1. Absorption spectra from 400 to 2498 nm of an empty sample cup with a black rubber back.

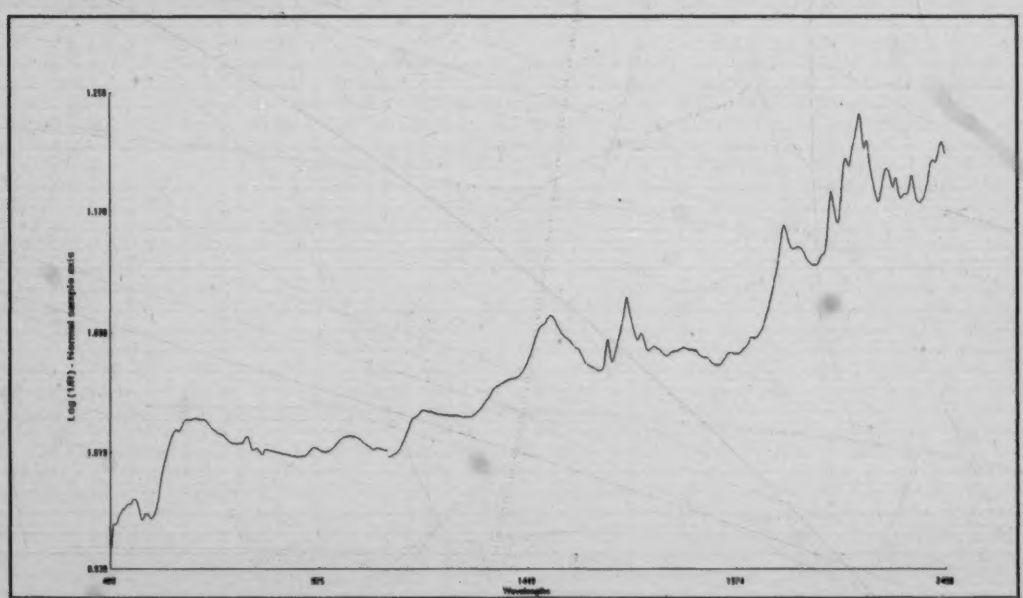

Figure 2.2. Absorption spectra from 400 to 2498 nm of the same sample cup containing 5 mg of pure DON.

Absorption spectra of DON were revealed through a difference scan using the GRAPHICS option of WINISI II by taking the spectra of the sample cup containing the 5 mg of DON and subtracting the scan of the empty sample cup (Figure 2.3). The difference graph shows an absorption scan similar to the previous scan of the DON, but with enhanced peaks making it easier to determine the ranges that calibration should be based on.

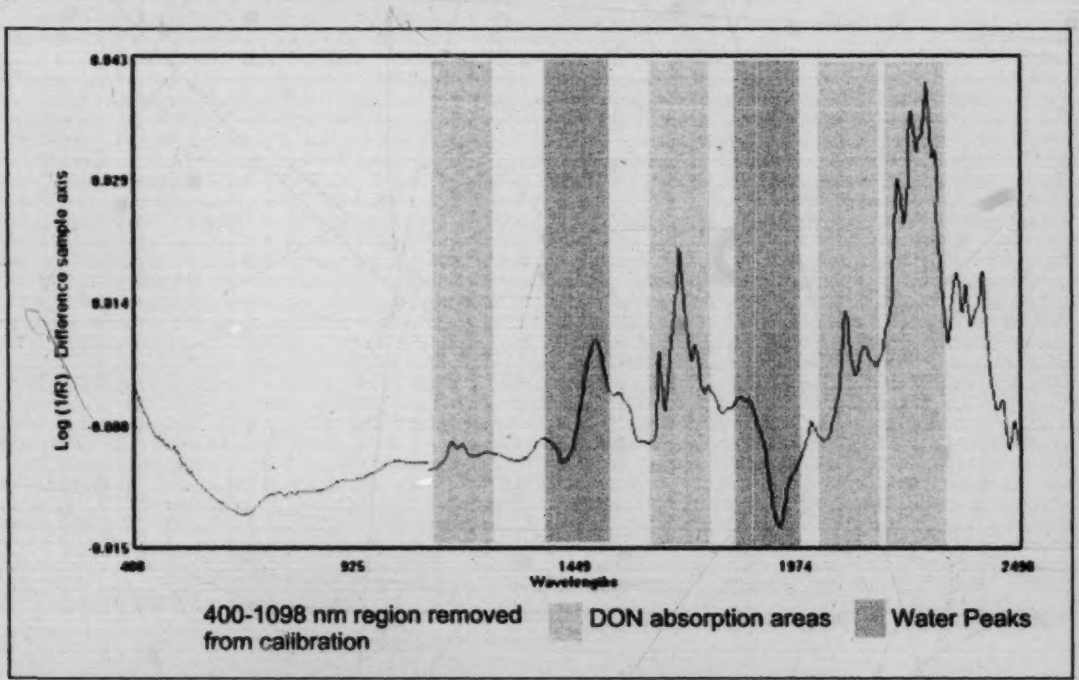

Figure 2.3. Absorption spectra of DON from 400 to 2498 nm.

Removal of the visible region of the spectrum (400 to 700 nm) will eliminate any influence that seed discoloration or fungi color might have on the calibration. Since discernable peaks did not exist in the region of 700 to 1098 nm, all the wavelengths from 400 to 1098 nm were eliminated. Identification of the 400 to 1098 nm region uses one detector and therefore makes a logical point

to divide the wavelength range. The spectra also show water peaks at 1450 and 1900 nm that were removed during wavelength selection. After eliminating the lower region and the water peaks, four peaks were visually identified as possible target areas; 1100 to 1200, 1670 to 1720, 2100 to 2150, and 2250 to 2350 nm.

Since there has been no published data on the absorption spectra of DON, it is difficult to compare the results if this study with other works in this area. The wavelengths selected in the 1100 to 1200 nm range are in agreement with Delwiche et al. (2005a), who evaluated the feasibility of using NIRS for the optical sorting of scab damaged kernels in wheat. Using 1150 and 1250 nm in the near infrared region, they were able to achieve 97% classification accuracy of damaged kernels; however, determining the concentration of toxin on the kernels was not mentioned. Wicklow et al. (2007) showed similar results when using NIR spectroscopy to sort corn kernels contaminated with mycotoxins. The wavelengths selected for their sorting technology were 500 nm, which is in the visible region, and 1200 nm, which was also included in this calibration model. While they were able to correctly classify 87% of the infected kernels, some kernels with only minor symptoms of discoloration had very high contamination levels of toxin and this resulted in higher errors. This suggests that the discoloration in the kernels is negatively influencing the ability of NIRS to detect DON and the inclusion of wavelengths in the visible region is amplifying this effect.

Pettersson and Aberg (2003) also indicated the negative influence of color and other physical damage on NIRS calibration. Their calibration model utilized

the wavelength range from 570 to 1100 nm to predict whole grain wheat samples; however, when samples containing more pink and shriveled kernels were scanned, they saw an increase in the standard error values due to the higher absorption in the visible region.

Elimination of the visible region should allow the calibration model to withstand the influence of color and physical abnormalities and focus on the actual DON content. While the 1100 to 1200 nm region has been utilized in previous studies for detection of toxins, the other three regions have not been previously identified as regions for DON absorption.

2.3.2 Calibration Development on the Dilution Set

Principle component analysis (PCA) was applied to the calibration set to study the structure of the data. A two-dimensional diagram of the first principle component (PC1) versus the second (PC2) principle component of the calibration data set, which together represents 88.62% of the data variance is shown in Figure 2.4.

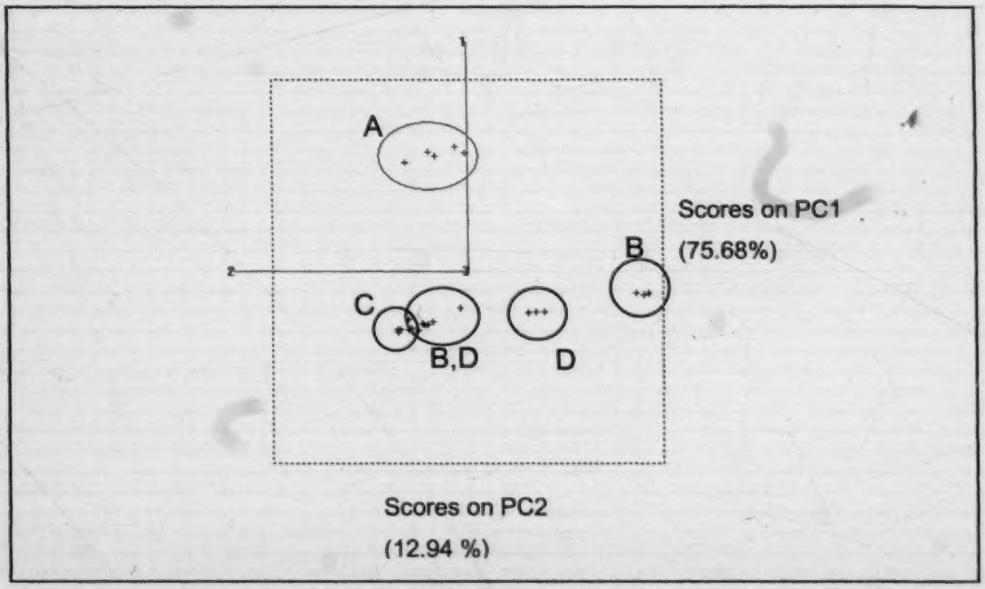

Figure 2.4. PCA classification of 24 ground wheat samples with 0% (A), 6.937% (B), 13.875% (C) and 27.75% (D) DON projected onto PC1 and PC2.

The largest component of the variation (75.68%) was explained by the difference between the pure flour samples and the samples that had been spiked with DON. The pure flour samples (A) cluster at the top of the box and formed a distinct group clearly separated from the other samples that contain DON. The second component that explained a further 12.94% of the variation was the concentration of DON within the calibration set of dilution samples. The different DON dilution sets (B, C, and D) form groups as indicated in figure 2.4. This gives an indication of how well the mixing was accomplished. Both the original set and the replicate set of standards containing 13.87% DON (C) form a single group indicating that both sets were similar in composition and were mixed properly. The sets of samples that contained 6.9% and 27.8% DON were not contained in a single group. Three samples of the 6.9% DON (B) were

grouped together, 3 samples of the 27.8% DON (D) formed another group and the other 6 samples (3 samples of 6.9% and 3 of 27.8%) formed a loose group together (B,D). This indicates that the two sets of samples from the other concentration groups are not as uniform as the samples from the 13.9% concentration group. Since the DON concentration for these samples was determined by calculation only, it was not possibe to determine which samples were closest to the calculated concentration, or if a real difference in DON concentration existed between the two sets. All of the samples were kept in the calibration set unless our calibration shows that there is too much difference within the sets to give an accurate equation.

Therefore, the calibration set for DON consisted of 24 samples with 27.8, 13.9, 6.9 and 0 ppm (pure flour) DON content. By looking at the absorption scans of the calibration set, it is possible to see variation between samples within the calibration ranges selected (Figure 2.5, A) and modifying the absorption data with a first derivative math treatment show emphasizes the peaks in the selected areas (Figure 2.5, B).

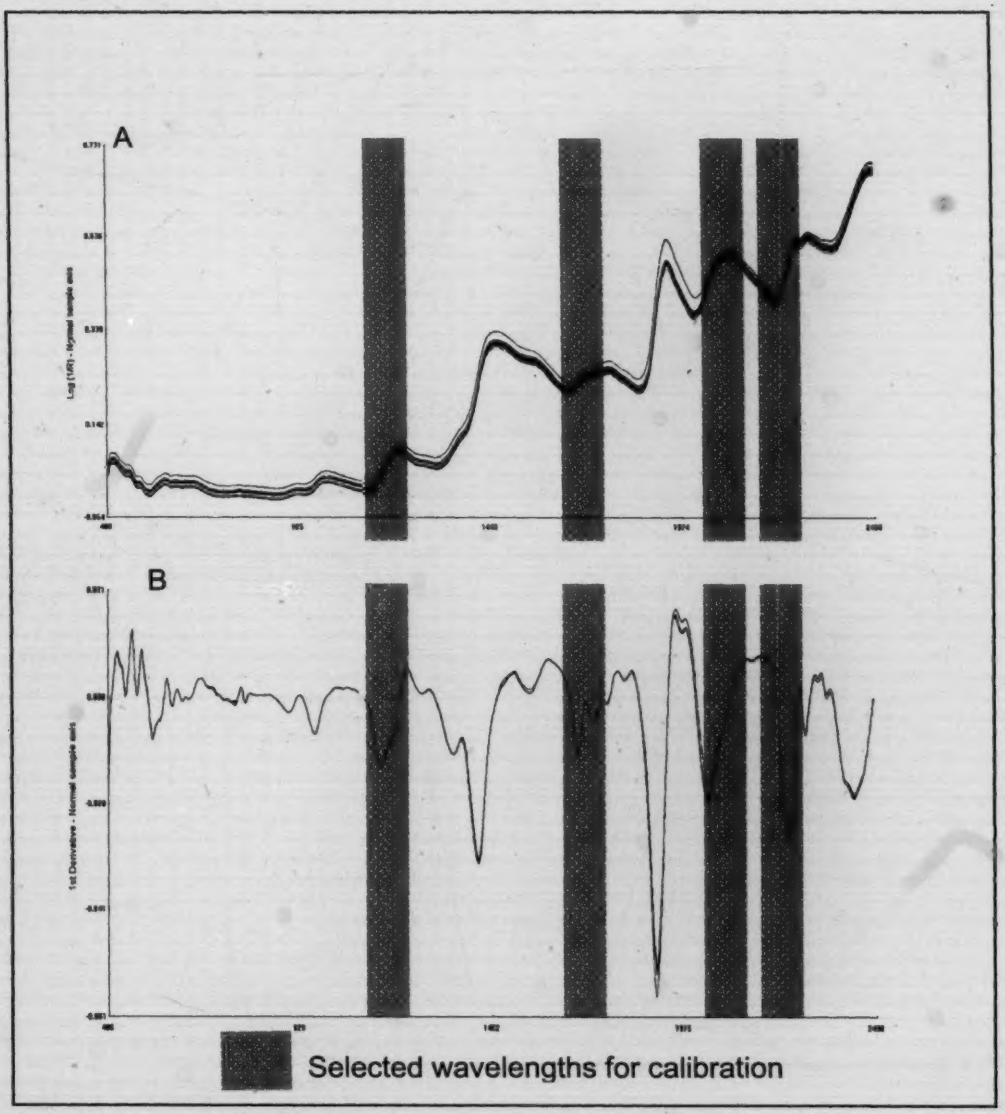

Figure 2.5. The absorption spectra (400 to 2498 nm) for the dilution samples using log (1/R) (A), and a first derivative math treatment (B).

As part of the wavelength selection, the areas selected for calibration were magnified to determine if the different concentrations could be visually separated from each other (Figure 2.6).

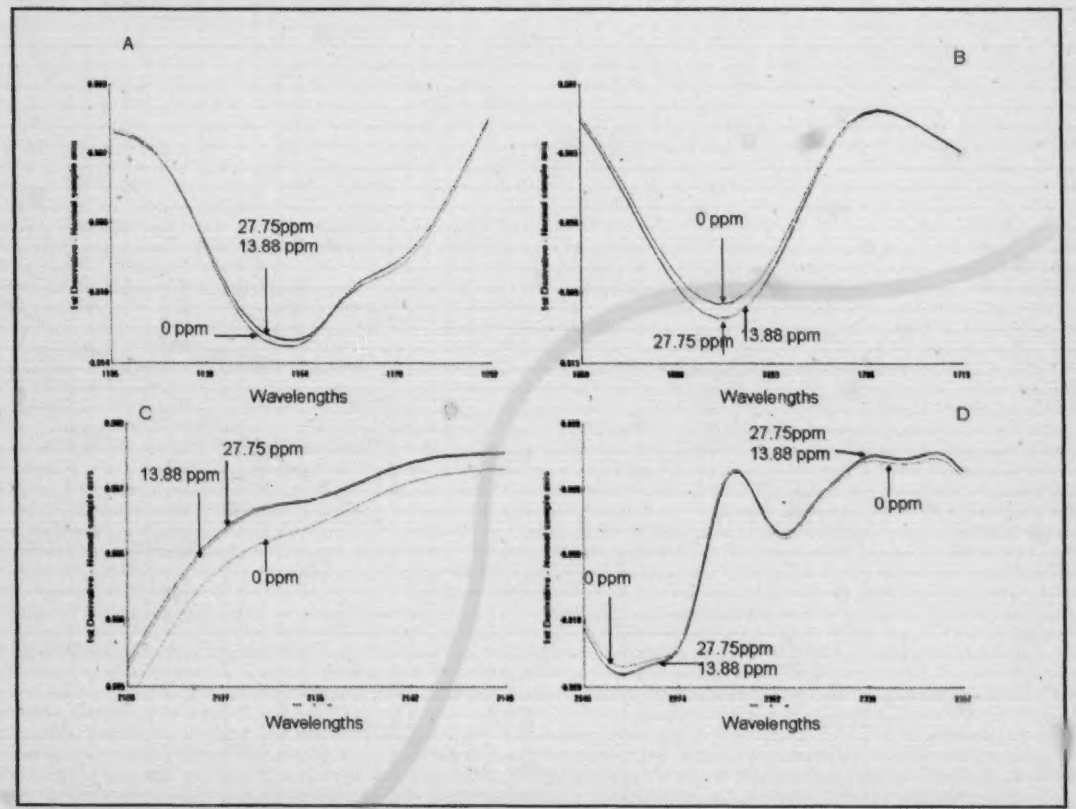

Figure 2.6. Wavelength ranges (A, 1100 to 1250 nm; B, 1670 to 1720 nm; C, 2100 to 2200; D 2250 to 2350 nm) magnified to show the variation between the standards with different DON concentrations.

In all regions the standards containing DON are clearly distinguished from the samples containing pure flour indicating that the wavelengths chosen were affected by the DON present in the sample. Calibrations were developed using MPLS regressions to predict DON concentration using the full wavelength range

and the modified wavelength range to compare results. The coefficient of determination (R^2), standard error of calibration (SEC) and the standard error of cross validation (SECV) are presented in Table 2.1 for the seven different calibration equations developed. Equation 1 was developed from the full wavelength range from 400 – 2498 nm, using every 16 nm which is the default setting in the WINISI software. Equation 2 was developed using only the four wavelength ranges selected for specific DON absorption, again using the software default of every 16 nm. Equation 3 has the same wavelength range as equation 2, only it is developed using every 4 nm instead of every 16 nm. Equations 4 to 7 were developed to test the wavelength range selected. To test whether the ranges were contributing to the prediction of DON or if they were redundant, each one of the four wavelength ranges was eliminated systematically and the NIRS recalculated.

Table 2.1. Statistical data for DON calibration models using different wavelength ranges.

	Wavelength Range (nm)	Total # Wavelengths	R^2	SEC	SECV
Equation 1	400 –1098 , 1100 - 2498 Every 16 nm	259	0.98	1.29	2.42
Equation 2	1100 –1250, 1670 –1720 2100 – 2200, 2250 - 2350 Every 16 nm	51	0.98	1.37	2.23
Equation 3	1100 – 1250, 1670 - 1720 2100 – 2200, 2250 to 2350 Every 4 nm	201	0.99	1.21	2.08
Equation 4	1100 – 1250, 1670 – 1720 2100 – 2200 Every 4 nm	150	0.99	1.22	2.01
Equation 5	1100 – 1250, 2100 – 2200 2250 – 2350 Every 4 nm	175	0.97	1.70	2.17
Equation 6	1100 – 1250, 1670 - 1720 Every 4 nm	99	0.97	1.75	2.61
Equation 7	1670 – 1720, 2100 - 2200 Every 4 nm	77	0.96	2.04	3.57

R^2 Coefficient of determination

SEC = Standard Error of Calibration

SECV = Standard Error of Cross Validation

The first equation is the only one that uses spectral information collected from the entire range of wavelengths to develop the calibrations. With an R^2 of 0.98 and SEC of 1.29 ppm, this equation shows some improvement over equations developed by Ruan et al. (2002). Using a neural network and barley samples containing DON concentrations between 0.3 to 50.8

ppm, their calibration equation had a high R^2 value of 0.93, but a SEP of 3.097 ppm. This study was able to reduce the errors during calibration by eliminating samples that were extremely high in DON concentration, and therefore increase the accuracy of the calibration equation at the low concentration levels of DON.

The concern with using the full range of wavelengths is the influence that color and other physical characteristics will or could have on this calibration model. Samples with more pink and shriveled kernels will have higher absorption in the visible region, which can decrease the accuracy of the equation (Pettersson and Aberg, 2003). Eliminating the visible region should minimize components in the model not directly related to DON concentration.

Eliminating the visible region and focusing on the 4 regions with identified peaks, the resulting equation (equation 2) has an R^2 equal to the equation developed on the full spectrum range (0.98), a slightly higher SEC (+ 0.08 ppm) but a lower SECV (-0.19 ppm). The removal of 208 wavelengths had very little impact on the calibration equation, which indicates that the correct wavelengths were selected to characterize DON absorption.

Equation 3 was developed using the same wavelength ranges as equation 2, but the spectral gap was reduced to every 4^{th} nm thereby increasing the number of wavelengths used from 51 to 201. From our experience a spectral gap of 16 nm, which is the default setting for calibration using WINISI, has provided good results for previous calibration development. However, since such large areas of the spectrum were removed from the calibration model, increasing the number of wavelengths in the spectral regions could better define

the peaks and result in an equation that would be more precise. Equation 3 shows a similar R^2 value of 0.99, and both the SEC and SECV are slightly lower than equation 2 (-0.16 and –0.15 ppm respectively). This indicates that increasing the number of wavelengths in the spectral regions of interest has resulted in a slightly more accurate equation.

Four more calibrations were developed that included variations on the 4 original peaks (equations 4 to 7). Elimination of the 2250 to 2350 nm range (equation 4) decreased the number of wavelengths to 150; however, it had little influence on the R^2, SEC or the SECV. This indicates that this wavelength range is redundant and can be removed from the calibration model.

When any of the other wavelength ranges were eliminated from the model (equations 5 to 7) the equations developed were of inferior quality with lower R^2 and higher SEV and SECV values, indicating that the optimal calibration model is represented by equation 4 as illustrated in Figure 2.7.

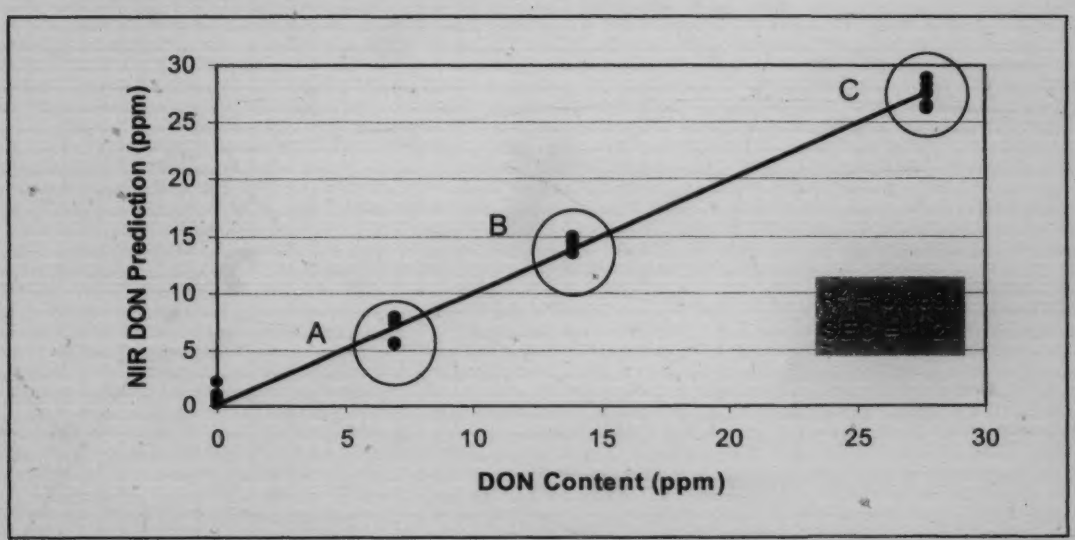

Figure 2.7. Optimal NIRS calibration equation for DON concentration using, 150 wavelengths from 1100 to 1250, 1670 to 1720 and 2100 to 2200 nm.

The developed calibration does show that there are some slight variations within the standards that were developed. The samples with 27.75% (C) and 6.937% (A) DON show some variability in their NIRS prediction values (Figure 2.7), and the groups are not as tight as those containing 13.875% (B). This was previously indicated during the PCA analysis as a possible error during our calibration development. Remixing of the standards at these concentrations could have lowered the SEC and developed a slightly better equation in terms of lowering the error.

With very few studies published in the literature about the development of NIRS for DON analysis, these results are difficult to compare. Previous studies that utilized the complete wavelength range to develop equations produced similar results. Calibrations developed by Petterson and Aberg (2003) had a R^2 value of 0.984 but a lower SECV of 381 ppb. As discussed earlier, fixing the variation within our calibration samples would reduce the error of our calibration and bring it closer to Petterson and Aberg's results of 381 ppb. However when samples that were highly discolored and containing a higher number of kernels showing abnormalities due to fungal infection were added to the calibration, the R^2 values decreased to 0.90 and the SECV values increased to 1234 ppb (Pettersson and Aberg, 2003). Having eliminated the visible wavelength region, the calibration developed in this study should be able to ignore changes in color and physical characteristics and focus on the amount of DON present for its analysis.

Of the previous studies examined, Wicklow et al (2007) and Delwiche et

al. (2005a and 2005b) used specific wavelengths to develop NIRS calibrations for DON. Wicklow et al. (2007) used two wavelengths to identify aflatoxins in corn, 500 and 1200 nm, of which 500 nm is in the visible region. This would strongly influence their equation to detect color, limiting the accuracy of the calibration equation to the relationship between toxin development and fungal infection.

Calibration equations developed by Delwiche et al. (2005a) were able to classify scab damaged kernels with 94% accuracy. However, when expanding their research to also look at DON levels, the results were more variable. Using wavelengths of 675 and 1480 nm, the DON level in the sorted wheat compared to that in the unsorted wheat ranged from 18 to 112%, with an average of 51%. Again the inclusion of wavelengths in the visible region is hampering the detection of DON.

While the inclusion of wavelengths in the 1100 to 1200 nm region is supported by other studies in literature, there is no supporting information on the other wavelengths used in this study. Comparison between our developed equations show that reducing the wavelengths in the NIRS calibration had very little effect on the predictability of DON, suggesting that we have successfully characterized DON absorption with only 150 wavelengths.

2.4 Conclusions

This project has been able to isolate wavelengths specific for DON absorption and use them to develop a calibration equation specific for the toxin. With only three wavelength ranges (1100 to 1250, 1670 to 1720, and 2100 to 2200 nm) the NIRS equation had a high R^2 value (0.99) while maintaining a SEC value of 1.2 ppm. Using only wavelengths that are specific for DON content, the calibration equation developed should be unaffected by physical abnormalities caused by fungal infection. Although the SEC of our calibration is still higher than our targeted range of less than 1 ppm, it shows that NIRS can be used as a tool for DON analysis. The next step in finalizing this equation is to use naturally-infected samples of grain to test our wavelength selection and expand our calibration. Using naturally-contaminated samples and precise laboratory analysis will develop a robust equation that can be used for routine analysis of DON in grain samples.

2.5 References

Bai,G.H., R. Plattner, A. Desjardins and F. Kolb. 2001. Resistance to *Fusarium* head blight and deoxynivalenol accumulation in wheat. Plant Breed. 120:1–6.

Cromey, M.G., S.C. Shorter, D.R. Lauren, K.I. Sinclair. 2002. Cultivar and crop management influences on *Fusarium* head blight and mycotoxins in spring wheat (Triticum aestivum) in New Zealand. N.Z. J. Crop Hort. Sci. 30:235–247.

Delwiche, S.R., C.S. Gaines. 2005a. Wavelength selection for monochromatic and bichromatic sorting of *Fusarium*-damaged wheat. Appl. Eng. Agric. 21(4):681-688.

Delwiche, S. R., T.C. Pearson and D.L. Brabec. 2005b. High-speed optical sorting of soft wheat for reduction of deoxynivalenol. Plant Dis. 89:1214-1219

Desjardins, A.E. 2006. *Fusarium* Mycotoxins: Chemistry, Genetics, and Biology. APS Press, St. Paul, Minnesota, USA.

Desjardins A.E., T.M. Hohn, S.P. McCormick. 1993. Trichothecene biosynthesis in *Fusarium* species: chemistry, genetics, and significance. Microbiol. Rev. 57: 595-604.

Fink-Gremmels, J. 1999. Mycotoxins: their implications for human and animal health. Vet. Quat. 21:115-120.

Miedaner, T., B. Schneider, and H.H. Geiger. 2003. Deoxynivalenol (DON) content and *Fusarium* head blight resistance in segregating populations of winter rye and winter wheat. Crop Sci. 43: 519–526.

Pettersson, H. and L. Aberg. 2003. Near infrared spectroscopy for determination of mycotoxins in cereals. Food Control. 14: 229-232.

Prelusky, D.B., R.G. Gerdes, K.L. Underhill, B.A. Rotter, P.Y. Jui, H.L. Trenholm. 1994. Effects of low-level dietary deoxynivalenol on haematological and clinical parameters of the pig. Nat. Toxins 2: 97–104.

Rotter B.A., D. B. Prelusky, J.J. Pestka. 1996. Toxicology of deoxynivalenol vomitoxin. J. Toxicol. Environ. Health 48:1-34.

Ruan, R., Y. Li and P. Chen. 2002. Non-Destructive Determination of Deoxynivalenol Levels in Barley Using Near-Infrared Spectroscopy. Appl. Eng. Agric. 18(5): 549-553.

Scott, P.M.. 1991. Possibilities of reduction or elimination of mycotoxins present in cereal grains. In J. Chelkowski (Ed.), *Cereal Grain. Mycotoxins, fungi and quality in drying and storage* Amsterdam: Elsevier. pp. 529-572.

Shenk, J., and M. Westerhaus. 1992. WINISI II Manual. Infrasoft Int., Port Matilda, PA, USA.

Wicklow, D.T., T.C. Pearson and D.L. Brabec. 2007. NIR spectroscopy as a tool for optimizing sorting of white corn kernels contaminated with mycotoxins. Corn Dry Milling Conference Proceedings. May, 2007.

Williams, P. and K. Norris (Eds.) 2001. Near-Infrared Technology in the Agricultural and Food Industries, 2nd edition. American Association of Cereal Chemists, Inc., St. Paul, Minnesota, USA.

Zhou, W.-C., Kolb, F.L., Bai, G.-H., Domier, J.-B., 2002. Effect of individual Sumai 3 chromosomes on resistance to scab spread within spikes and deoxynivalenol accumulation within kernels in wheat. Hereditas 137:81–89.

Chapter 3

Using Near Infrared Spectroscopy and Modified Wavelength Ranges to Predict Deoxynivalenol Levels in Naturally Infected Wheat and Barley

3.1 Introduction

The effects of Fusarium infestation and the consequent production of Deoxynivalenol (DON) in cereal crops have negatively impacted the agricultural industry as a whole. The lack of a quick and precise methodology to detect DON has further complicated the situation by preventing the industry to effectively manage infected grains.

There have been several studies looking at near infrared reflectance spectroscopy (NIRS) to bridge this gap. Segregation of *Fusarium* damaged kernels using NIRS has been successful, achieving as high as 97% classification accuracy (Delwiche et al., 2005a; Wicklow et al., 2007). However, separation and removal of the visually infected kernels do not conclusively reduce the amount of DON present in the sample. In a study by Delwiche et al. (2005b) the amount of DON level in the sorted wheat compared to that in the unsorted wheat ranged from 18 to 112%, with an average of 51%.

The inconsistency between fungal infection and toxin formation have been well documented (Cromey et al., 2002; Zhou et al., 2002; Miedaner et al., 2003)

and could be causing difficulties in obtaining a precise NIRS equation. Pettersson and Aberg (2003) concluded that factors such as discoloration and shriveled kernels were interfering in the calibration of their near infrared transmission (NIT) equation for DON. While studies have shown that the potential to use NIRS for DON prediction is there, an equation that can predict toxin levels without interference from other physical factors has yet to be developed.

The purpose of this study was to develop a NIRS method that will predict the amount of DON precisely without influence from physical characteristics of the grain. Naturally infected samples and specific wavelengths selected for DON absorption (1100 to 1200; 1670 to 1720; 2100 to 2200) were used to develop a NIRS calibration equation.

3.2 Materials and Methods

3.2.1 Samples

Four hundred and ninety two samples of barley and wheat were collected for this study. This set included barley samples collected from the *Fusarium* Head Blight testing nursery at the Agriculture and Agri-Food Canada station in Brandon, Manitoba (270 samples, 2004 crop year) and from Agriculture and Agri-food Canada station that were grown in Prince Edward Island and New Brunswick (52 samples, 2004 crop year). It also included a set ofwheat samples

collected from the Canadian Grain Commission in Winnipeg, MB (170 samples, 2004 crop year). All samples were ground to <1mm before being shipped to the Field Crop Development Centre (FCDC) in Lacombe.

Of the 492 samples collected, only 182 samples were selected for GC analysis. Samples were selected based on their spectral absorptions using WINISI II SELECT option (Infrasoft International LLC, Port Matilda, PA, USA) as well as using other data provided with the samples, such as severity of *Fusarium* infection and DON content determined by ELISA.

Twelve of the samples showed abnormalities during GC analysis and DON was not detected. Non detection during GC analysis usually occurs when the amount of DON present is below the detection limit of the instrument, but the level is not 0%. Reanalysis of these samples gave similar results, and since there was a high proportion of samples in 0 – 1ppm range already, the non-detected samples were eliminated from the calibration set. Thirteen outlying spectra with global outliers of H > 10.0 (Mehalanobis distance; Shenk and Westerhaus, 1992) were deleted from the calibration set before model development.

Sixteen of the remaining samples were randomly selected for a validation group leaving 141 samples for calibration. Of these 141 samples, 61 samples were wheat and 80 samples were barley. The validation set consisted of 9 samples of wheat and 7 samples of barley.

3.2.2 *NIRS Reflectance and Model Development*

All the samples for the study were scanned using a FOSS NIRSystems 6500 (Foss North America, Eden Prairie, MN, USA) spectrophotometer equipped with a transport module. A quarter size sample cup (46 X 46 mm) was used with a total surface area of 21.16 cm^2 for spectra collection. Approximately 5 g of each sample was poured into the sample cup, excess sample was scraped off to distribute the sample evenly and the lid pressed onto the back. The instrument collected 32 scans per sample that were averaged to produce 1 spectrum (Shenk and Westerhaus, 1992). Data for each spectrum was collected from 400 to 2500 nm at 2 nm intervals using the integrated software WINISI II (FOSS NIRSystems Inc., Silver Spring, MD, USA) for 1050 absorption readings per sample. Absorption was recorded as log of 1/reflection (Shenk and Westerhaus, 1992).

3.2.3 *Development of Calibration Equations*

Spectral information was subjected to mathematical procedures using WINISI II calibration software. Calibration models were developed over the range of 400 to 2498 nm, as well as a modified wavelength range determined to be specific for DON absorption (1100 to 1250, 1670 to 1720, 2100 to 2200 nm) for comparison. The best combination found after evaluating many different derivatives and regression algorithms was a modified partial least squares

(MPLS) math treatment (Shenk and Westerhaus, 1992) using the first derivative, calculated over a 4 point (8 nm) gap and a 4 point (8 nm) smooth (1,4,4,1 software setup). The software was setup for 8 cross validation groups and all calibrations developed used the same math treatment and setup.

3.2.4 Gas Chromatography with Electron Capture Detection

3.2.4.1 Sample Preparation

The analysis of the samples by GC was performed similar to the procedure described by Tacke and Casper (1996). Five g of ground barley was extracted with 40 ml of 84% acetonitrile for 60 min on a horizontal shaker. The contents were allowed to settle for 5 min in a vertical position. Four ml of supernatant was added to a Al/C_{18} clean-up column and allowed to drip completely through the column. Two ml of the eluant was transferred to a test tube (13 x 100 mm) with Teflon-lined screw cap. The contents of the test tube were evaporated to complete dryness at 55°C under a moderate flow of nitrogen purge gas (approximately 30 min). One hundred µl of trimethylsilyimidazole :trimethylchlorosilane mixture (9:1) was added to the dried residue and allowed to stand at room temperature for 10 min. One ml of mirex (0.05 mg/l) was added, the mixture was vortexed for 10 sec and then 1 ml 3% $NaHCO_3$ was added and mixed well. The solution was allowed to stand until the upper organic layer was clear (approximately 1 min) and then the upper portion was transferred to a GC vial. One µl was injected in split-less mode for GC/ECD analysis.

3.2.4.2 GC Apparatus

Separation and detection were performed using a Hewlett-Packard 6890 Series Gas Chromatograph with HP ECD System (Hewlett Packard, Waldbronn, Germany) equipped with an electron capture detector and a front split/splitless injector inlet. The GC operated in the splitless mode with helium as the carrier gas at a flow rate of 4.7 ml/min and nitrogen as detector gas with a flow of 30 ml/min. The injector and detector temperatures were adjusted to 250°C and 330°C, respectively. The analytical column used was a capillary column (Model Aglient 123-3832, 30 m x 0.32 mm, 0.25 µm, Hewlett-Packard, Waldbronn, Germany). The detection limit of this instrument is 0.1 to 0.2 ppm.

3.3 Results and Discussion

The DON content in the calibration set ranged from 0 to 10.9 ppm. However, a high frequency of samples contained 0 to 0.2 ppm DON and samples for the range from 7.4 to 10.9 ppm DON were lacking (Figure 3.1). Typically, uniform distribution of samples for NIRS calibration is preferred to eliminate any bias the equation has towards one highly represented group; however, since the primary interest is in the extremely low concentration range of DON, the higher proportion of samples in this area should not adversely affect the NIRS equation and therefore were left in the calibration group. Obtaining more samples to fill in the gap at the higher concentration range from 7 to 10 ppm will be necessary to develop a more robust equation.

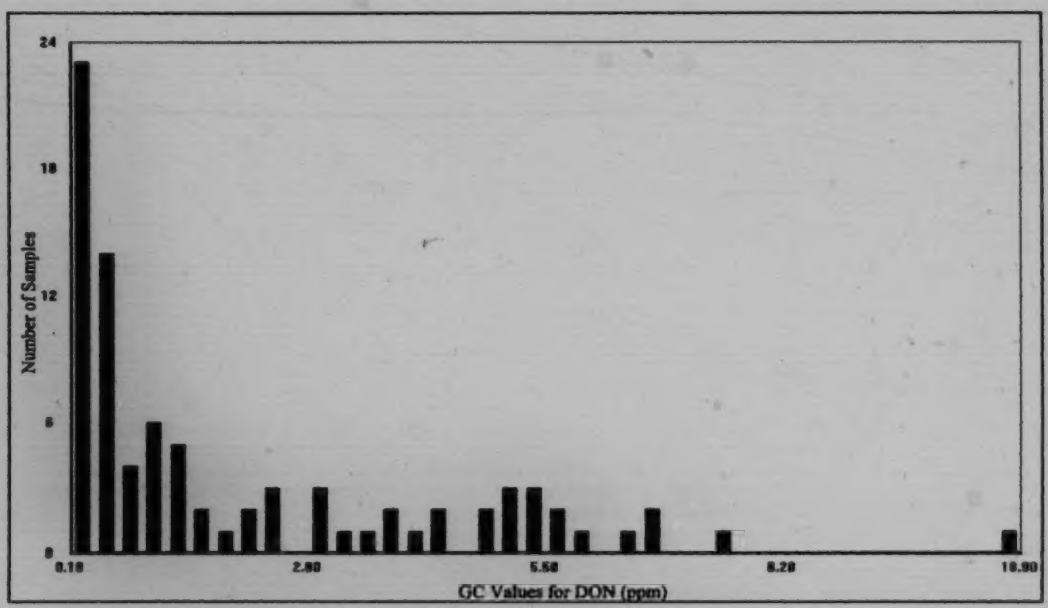

Figure 3.1. Distribution of calibration samples based on DON content determined by GC analysis.

For this calibration both wheat and barley samples were included. In the calibration group there were 61 samples of wheat with a range of DON content from 0 to 2.0 ppm. The range of DON concentration for the calibration group is due to the 80 barley samples within the set that varied from 0 to 10.9 ppm. The wheat samples incorporated in the calibration would expand the model so that one equation would be able to predict DON content in both crops.

The absorption scans of the 141 samples used for calibration are shown in Figure 3.2. There was a wide range of variation among the scans, a large portion of which is the direct result of inclusion of both wheat and barley in the calibration group.

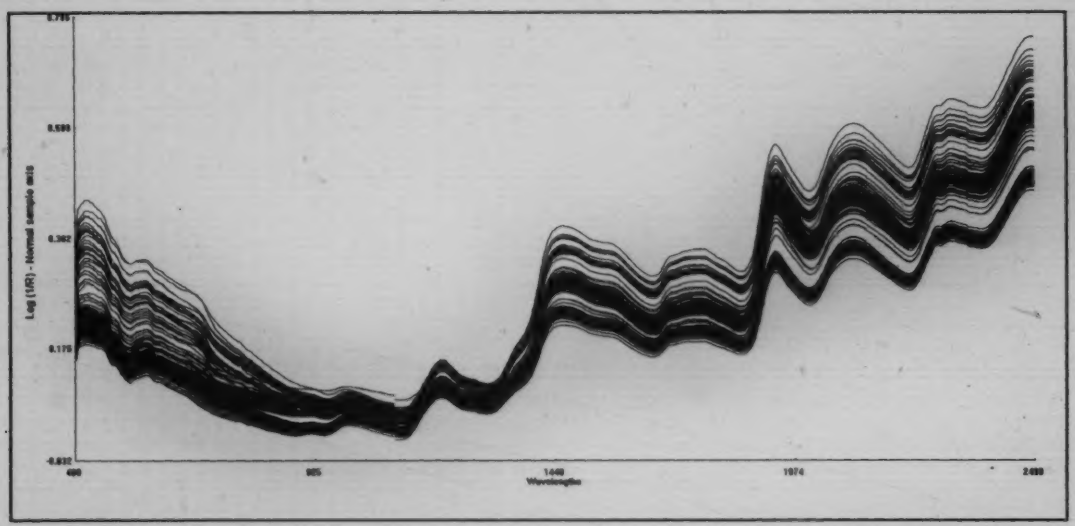

Figure 3.2. Absorption scans of the 141 samples used for DON calibration.

By looking at the absorption scans of the calibration set, the barley scans are not distinguishable from the scans of wheat. This blending of crops within the calibration set is partially due to the fact that all samples were ground. Since the samples are not segregating into groups, this suggests that the samples will fit onto the same calibration curve.

The coefficient of determination (R^2), standard error of calibration (SEC) and the standard error of cross validation (SECV) for the four developed equations are presented in Table 3.1 and the equations developed are illustrated in Figure 3.3.

Table 3.1. Summary of calibration statistics for modified PLS models using full and reduced wavelengths.

Equation	Wavelength Range	# Samples	# Wavelengths	Min (ppm)	Max (ppm)	SEC	R^2	SECV
1	400 - 2498,16 nm	141	259	0.00	10.80	0.63	0.91	0.69
2	400 - 2498, 4 nm	141	1034	0.00	10.80	0.63	0.91	0.69
3	1100-1250; 1670-1720; 2100-2200,16 nm	141	38	0.00	10.80	0.66	0.90	0.76
4	1100-1250; 1670-1720; 2100-2200, 4 nm	141	150	0.00	10.80	0.64	0.90	0.72

R^2 Coefficient of determination, SEC – Standard Error of Calibration

SECV – Standard Error of Cross Validation

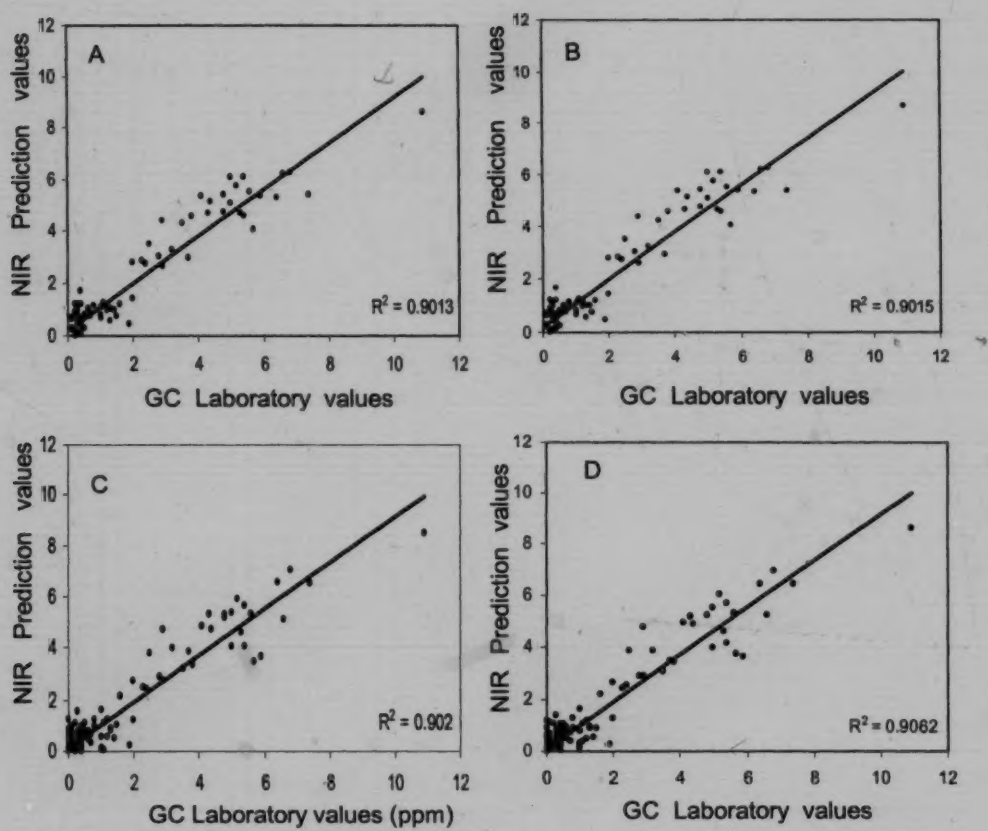

Figure 3.3. Calibrations developed for DON content for equation 1 (A), equation 2 (B), equation 3 (C) and equation 4 (D).

The full wavelength range from 400 – 2498 nm was used to develop equation 1 (Figure 3.3, A) using every 16 nm which is the default setting in the WINISI software. The same wavelength range was used to develop equation 2 (Figure 3.3, B) but the number of wavelength points were increased to 1034 by utilizing every 4th nm, to determine if it would increase the accuracy of the equation. These two equations were identical in their performance and had

an R^2 of 0.91, SEC of 0.63 ppm, and a SECV of 0.69 ppm. Therefore, the increase in wavelengths did nothing to improve the precision of the equation and is unnecessary if the full wavelength range for DON calibration is used.

The modified equation range selected specifically for DON absorption (1100 to 1250, 1670 to 1720, and 2100 to 2200 nm) was used to develop both equation 3 and 4 (Figure 3.3, C and D). Every 16^{th} nm was used for equation 3 and every 4^{th} nm was used for equation 4, which increased the number of wavelengths in equation 4 from 38 to 150, respectively (Table 3.1). While the R^2 of both equations was 0.90, the SEC values of equation 4 decreased by 0.02 and the SECV values decreased by 0.04. This agrees with our previous study using pure DON and commercial flour dilutions. While this change is very small, it may be useful especially in a calibration that is trying to predict such small concentrations of toxin.

The equations developed are comparable to other studies in this area. Ruan et al. (2002) developed an equation for DON using neural networks with an R^2 of 0.93; however, their SEP of the calibration was over four times higher at 3.097. Their study included barley from 0.3 to 50.8 ppm, which is well beyond the range needed for determining DON for industry requirements. Limiting their calibration to the lower concentrations of DON may have allowed them to lower the prediction error of their calibration. Detection of an analyte at high concentrations can cause a nonlinear response of the curvature type (Williams and Norris, 2001). This is not unusual and some curvature is expected in all instrument responses. While 50.8 ppm does not usually constitute a 'high

concentration', when compared to samples containing 0.3 ppm, it represents over 50 times the concentration.

Petterson and Aberg (2003) developed an NIT equation to predict DON with an R^2 value of 0.984 and a SECV of 381 ppb, which on the surface seems better than the calibration equation developed in this study. However, when their calibration was expanded with a set of samples that had a higher concentration of pink, shriveled kernels and was more visibly infected, the R^2 values decreased to 0.90 and the SECV values increased to 1234 ppb. Their expanded equation has an R^2 value very similar to that obtained in this study, but the error of their equation is higher than this studies result at 0.69 ppm. The addition of samples with the physical abnormalities caused their equation to change significantly. Selection of specific wavelengths for DON absorption should prevent the NIRS equation from breaking down when subjected to samples of higher infection.

Whereas the equations developed with the full spectrum look marginally better than those developed with the modified range, the concern would be the influence of the visible region on the NIRS prediction. Samples with pink and shriveled kernels have increased absorption in the visible region and have resulted in decreased R^2 values and increased SECV values in previous studies (Petersson and Aberg, 2003). There were extremely slight color variations within our calibration set. While there were some samples in our calibration group that had visible damage from *Fusarium* infection, these physical characteristics were lost when the samples were ground. In samples that had higher degrees of colorant or staining and in whole grain samples, the influence of the visible

region would increase and could cause problems.

3.3.1 Validation

The above calibrations were validated by randomly removing 16 samples from the calibration set prior to calibration, and then predicting these samples using the developed equations. Results from the validation set are listed in Table 3.2 and are illustrated in Figure 3.4.

Table 3.2. Summary of validation statistics for modified PLS models using full and reduced wavelengths for calibration

Equation	Parameters (range(s) and interval)	N	Min (ppm)	Max (ppm)	SEP	R^2	Slope
1	400 - 2498; 16 nm	16	0.00	7.10	0.65	0.95	1.181
2	400 - 2498, 4 nm	16	0.00	7.10	0.65	0.95	1.183
3	1100 - 1250, 1670 - 1720, 2100 - 2200; 16 nm	16	0.00	7.10	0.74	0.92	1.144
4	1100 - 1250, 1670 - 1720, 2100 - 2200; 4 nm	16	0.00	7.10	0.76	0.91	1.126

R^2 - Coefficient of determination, SEC – Standard Error of Calibration

SECV – Standard Error of Cross Validation

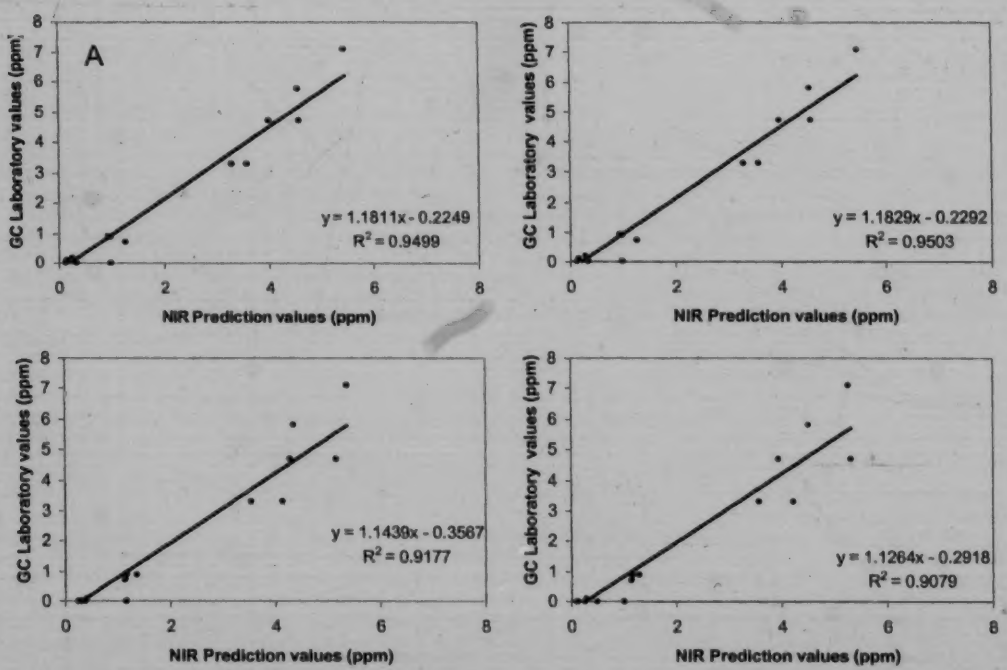

Figure 3.4. Validation of the developed equations using equation 1 (A), equation 2 (B), equation 3 (C) and equation 4 (D).

In the validation group, there was one sample that had a DON concentration of 7.10 ppm. By looking at the distribution of the calibration group (Figure 3.1) it is easy to establish that this sample is not well represented in the calibration, and it results in a higher residual error (1.8 ppm). Elimination of this single sample from the validation group, did not change the R^2, or SEP values appreciably, but it did decrease the slope of the prediction bringing it closer to the ideal slope of 1 (Figure 3.5).

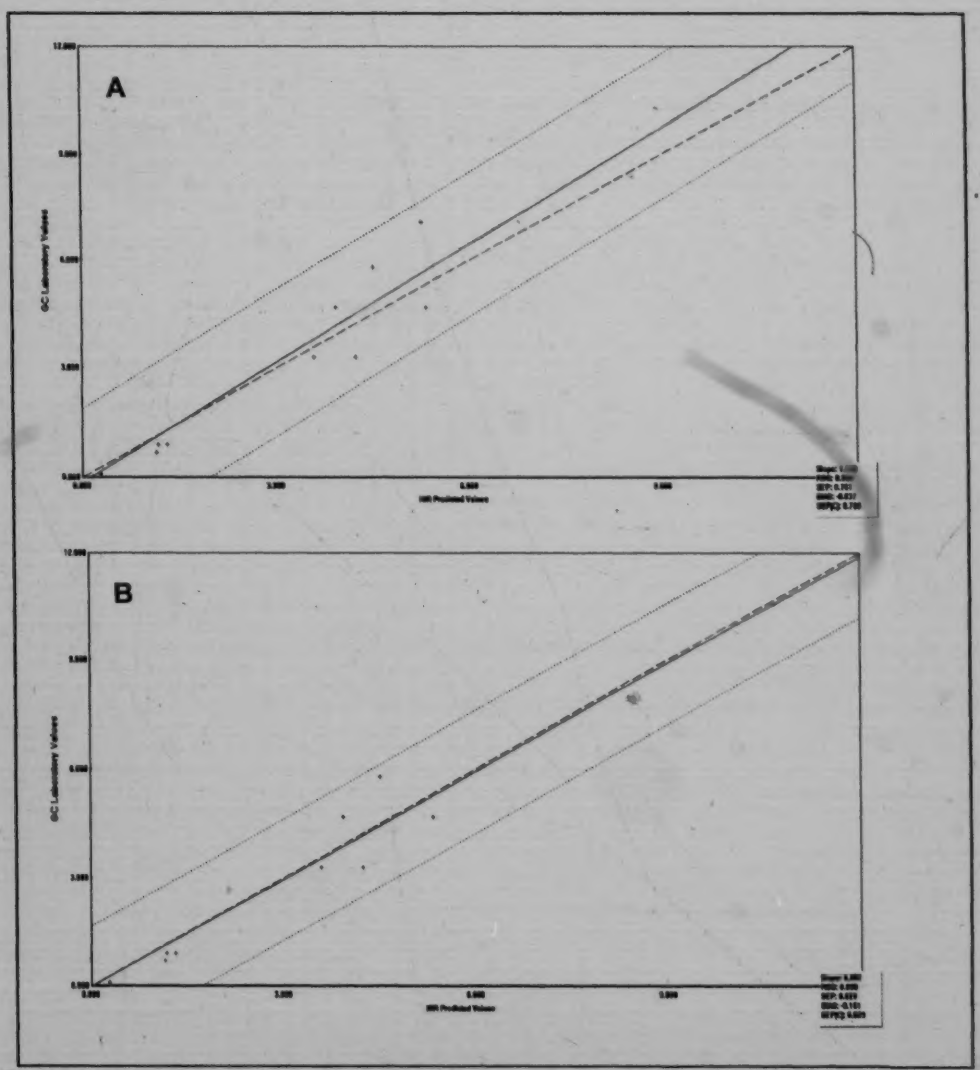

Figure 3.5. Prediction of the validation group using equation 4; before (A) and after (B) removal of the one sample containing 7.10ppm DON concentration.

Although the validation group was predicted very well with all equations, the performance was better for the equations that utilized the entire wavelength range. Had there been more discoloration in the samples, the difference in performance between the full wavelength equations and the modified

wavelength range may have been more evident.

After looking at the performance of both the calibration equations and the validation groups, this study was able to determine that using NIRS to predict DON content in wheat and barley is a viable option for plant breeders and industry groups. The high performance of the reduced wavelength equations show that enough of the region has been identified to characterize DON absorption without including redundant wavelengths in the calibration. More samples should be added to fill some of the gaps in the calibration groups and thereby increase the prediction capabilities.

The samples used for this study unfortunately did not have enough visible *Fusarium* damage and seed discoloration to rigorously test the modified wavelength range. Higher levels of color or damage would have allowed us to conclusively determine if using modified wavelength ranges would eliminate any influence that the *Fusarium* damage has on NIRS calibrations.

The next step in this program should be to collect samples with extremely high levels of visually apparent *Fusarium* damage to further test the equations. The modified wavelength range should also be applied to whole grain samples and test the results. If whole grain samples are used for calibration, *Fusarium* damage will be more evident on the intact grains, and the reduced wavelength ranges will be even more crucial to eliminate these effects.

3.4 Conclusions

The equations developed in this study effectively demonstrate that NIRS can be used to estimate DON content in wheat and barley. The equations that were developed on the full wavelength region and those developed using wavelengths specifically for DON absorption both show high R^2 values and SEC values that will be acceptable for industry requirements. Continuation of this project will add samples to the calibration group to fill in any gaps in the DON concentration range to make the equation more robust. The next step should be transferring this technique to whole grain samples and testing the performance.

3.5 References

Cromey, M.G., S.C. Shorter, D.R. Lauren, K.I. Sinclair. 2002. Cultivar and crop management influences on *Fusarium* head blight and mycotoxins in spring wheat (Triticum aestivum) in New Zealand. N.Z. J. Crop Hort. Sci. 30:235–247.

Delwiche, S.R., C.S. Gaines. 2005a. Wavelength selection for monochromatic and bichromatic sorting of *Fusarium*-damaged wheat. Appl. Eng. Agric. 21(4):681-688.

Delwiche, S. R., T.C. Pearson and D.L. Brabec. 2005b. High-speed optical sorting of soft wheat for reduction of deoxynivalenol. Plant Dis. 89:1214-1219

Desjardins, A.E. 2006. *Fusarium* Mycotoxins: Chemistry, Genetics, and Biology. APS Press, St. Paul, Minnesota, USA.

Miedaner, T., B. Schneider, and H.H. Geiger. 2003. Deoxynivalenol (DON) content and *Fusarium* head blight resistance in segregating populations of winter rye and winter wheat. Crop Sci. 43: 519–526.

Pettersson, H. and L. Aberg. 2003. Near infrared spectroscopy for determination of mycotoxins in cereals. Food Control. 14: 229-232.

Ruan, R., Y. Li and P. Chen. 2002. Non-Destructive Determination of Deoxynivalenol Levels in Barley Using Near-Infrared Spectroscopy. Appl. Eng. Agric. Volume 18(5): 549-553.

Shenk, J., and M. Westerhaus. 1992. WINISI II Manual. Infrasoft Int., Port Matilda, PA, USA.

Tacke, B.K. and H.H. Casper. 1996. Determination of Deoxynivalenol in wheat, Barley and Malt by Column Cleanup and Gas Chromatography with Electron Capture Detection. Journal of AOAC International. 79: 472 – 475.

Wicklow, D.T., T.C. Pearson and D.L. Brabec. 2007. NIR spectroscopy as a tool for optimizing sorting of white corn kernels contaminated with mycotoxins. Corn Dry Milling Conference Proceedings. May, 2007.

Williams, P. and K. Norris (Eds.) 2001. Near-Infrared Technology in the Agricultural and Food Industries, 2nd edition. American Association of Cereal Chemists, Inc., St. Paul, Minnesota, USA.

Zhou, W.-C., Kolb, F.L., Bai, G.-H., Domier, J.-B., 2002. Effect of individual Sumai 3 chromosomes on resistance to scab spread within spikes and deoxynivalenol accumulation within kernels in wheat. Hereditas 137:81–89.

Chapter 4

Project Summary

As long as current agricultural practices cannot prevent *Fusarium* infection of small grains, DON will continue to be a critical issue in animal and human health. All areas of the agriculture industry are adversely affected by DON contamination, causing wide spread economic repercussions. All animal species evaluated in literature have shown adverse effects from DON ingestion or inhalation, causing lower productivity, reproduction failures, altering immune function, and in severe cases, animal mortality (Prelusky et al., 1994). Many countries, including Canada, have regulated the amount of DON in grain products at maximum tolerable levels. Canadian guidelines for the maximum concentration of DON allowed in animal feed is 5 ppm for beef cattle, sheep and poultry; but only 1 ppm for swine, dairy cattle and horses (Manness, 2002). Because of near zero tolerance for DON, grain buyers and processors tend to avoid areas that have high incidence of the toxin causing a devastating effect on grain producers when they are unable to sell their crops (Wu, 2007). Development of a quick and precise method to detect DON would allow the industry to effectively manage infected grain and therefore limit the amount entering into the food chain.

Therefore, the present thesis investigated the ability of NIRS to precisely detect the concentration of DON in ground wheat and barley samples. Using a novel approach of wavelength selection and calibration development, a calibration was developed that will be able to detect DON at concentration

levels called for by the agriculture and food industries.

Previous works in this area have shown the potential to develop NIRS for detecting toxins (Ruan et al., 2002; Pettersson and Aberg, 2003; Delwiche et al., 2005a, b; Wicklow et al., 2007); however, the influence of color or physical characteristics of *Fusarium* infected grain created obstacles that prevented the development of a NIRS calibration capable of truly detecting DON content. Using a novel approach of wavelength selection, and a pure sample of the toxin, this study was able to produce a spectrum of DON. By visual selection, four sections of the NIRS spectrum were identified as important for DON absorption; 1100 to 1200, 1670 to 1720, 2100 to 2150, and 2250 to 2350 nm. Most importantly, the wavelengths from 400 to 1098 nm that include all of the visible spectral regions were removed to eliminate the influence of color and physical characteristics on our calibration development. Further testing proved the 2250 to 2350 nm region was not needed and thus this range was also eliminated. Calibrations developed on the reduced wavelength range were comparable to equations developed using the full wavelength range, indicating that the spectral information needed for a precise calibration had been captured using only the three ranges.

While our success using standard samples was encouraging, previous experience has shown us that calibrations developed on artificially created samples, rarely hold up to the analysis of natural samples, so it was necessary to transfer this model onto naturally-infected grains. The standard samples were created using white commercial flour, so that the calibration could focus on the

DON content without interference from other components such as variation in protein, starch and fiber. Naturally-contaminated samples not only have more variation in composition, but these samples will also likely contain discoloration and even more types of toxins, rather than just DON.

Using 141 samples of ground wheat and barley as well as the previously selected wavelength regions, a NIRS calibration was developed that had a high R^2 value of 0.90 and a SEC value of 0.64 ppm that is within industry standards for DON detection. The developed equation showed similar results to the equation developed on the full wavelength range, which indicated that all the spectral information needed to classify DON was included, while removing many of the wavelengths that could have been affecting previous attempts of using NIRS for this purpose.

While this study has been successful in achieving its goal of DON prediction by NIRS, some areas can be improved.

1. The wheat and barley samples collected for the calibration group had a limited range of DON content from 0 to 10.80 ppm, with a noticeable lack of samples in the 7 to 10 ppm range. To create a more robust calibration, more samples need to be added to expand the overall range of DON concentration as well as fill the gap at the 7 to 10 ppm range.

2. More work should be done to compare the errors of the GC analysis and sampling errors. When dealing with components in quantities this small, there is a need to have precise measurements throughout the methodology. It would be useful to have GC analysis done by another lab in addition to our work for

comparison. This would give another set of data to compare lab errors against. Lowering the errors of the calibration should continue to be a priority in the expansion of the calibration.

3. There should be more work done identifying other mycotoxin compounds such as 3 AcDON and 15 AcDON. This would allow us to develop a more complete picture in terms of toxins present and any interference from these toxins in calibration development.

4. This calibration work should be expanded to other feedstuffs that are important to the agricultural industry.

5. This study utilized ground samples for all of our calibration work; however, a calibration based on whole grain samples would be more suitable for industry. Due to the variability of DON distribution within a sample, grinding the grain disperses the toxin throughout the sample and makes it more homogenous. Developing the calibration on ground grain first, gave the opportunity to choose wavelengths without having to overcome the unsystematic distribution of DON in the sample. However, grinding samples can present another source of error to our calibration. DON can transfer in the grinder to other samples, as well as the error associated with different particle sizes due to kernel hardness. While proper grinding may produce a more uniformly sized sample, there may be different weights associated with the particles that are easily separated out during packaging and storage. A 1 mm piece of hull, for example, may be much lighter than a 1mm piece of endosperm and may tend to separate out from the rest of the sample. This is especially important for our project because DON

contamination in grain is limited to the outer layers of the kernel, the hull and the aleurone layer, and this creates problems with distribution of DON throughout the ground sample as well. The separation effect, in addition to the extremely small quantities that are measured, makes it difficult to control the errors in sampling.

One of the main purposes of this study was to eliminate the influence of color and kernel deformities on the calibration. Ground samples were used in this project to achieve the homogeneity of DON throughout the sample and provide the best opportunity to develop a specific calibration for DON while limiting the errors in our calibration. However, by using ground samples we also masked the color differences and the shrunken kernels within the samples which were needed to show the uniqueness of our calibration.

The next step for this project should take this calibration model and wavelength selection and transfer it to whole grain determination. Whole grain analysis eliminates the need for grinding, speeds up the analysis time and will give industry the rapid analysis needed to manage infected grains effectively. With the background information that we have now developed, the transfer of this calibration model to whole grain should be easier to accomplish while maintaining the specificity and the low calibration errors.

The use of whole grain will test the influence of color and seed abnormalities on our calibration model and show the true potential of our calibration. While we needed to do the background testing to refine our methods, the whole grain samples is where the calibration model will gain its benefits.

References

Delwiche, S.R., C.S. Gaines. 2005a. Wavelength selection for monochromatic and bichromatic sorting of *Fusarium*-damaged wheat. Applied Engineering in Agriculture. 21(4):681-688.

Delwiche, S. R., T.C. Pearson and D.L. Brabec. 2005b. High-speed optical sorting of soft wheat for reduction of deoxynivalenol. Plant Dis. 89:1214-1219

Manness, G., T. Nicholson, and C. Nicolaou. 2002. Ethanol: made in Manitoba. Winnipeg, MB: Manitoba Energy Development Initiative.

Pettersson, H. and L. Aberg. 2003. Near infrared spectroscopy for determination of mycotoxins in cereals. Food Control. 14: 229-232.

Prelusky, D.B., R.G. Gerdes, K.L. Underhill, B.A. Rotter, P.Y. Jui, H.L. Trenholm. 1994. Effects of low-level dietary deoxynivalenol on haematological and clinical parameters of the pig. Nat. Toxins 2: 97–104.

Ruan, R., Y. Li and P. Chen. 2002. Non-Destructive Determination of Deoxynivalenol Levels in Barley Using Near-Infrared Spectroscopy. Applied Engineering in Agriculture. Volume 18: 549-553.

Wicklow, D.T., T.C. Pearson and D.L. Brabec. 2007. NIR spectroscopy as a tool for optimizing sorting of white corn kernels contaminated with mycotoxins. Corn Dry Milling Conference Proceedings. May, 2007.

Wu, F. 2007. Measuring the economic impacts of *Fusarium* toxins in animal feeds. Anim. Feed Sci. and Tech. 137 (2007) 363–374.